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HEAT-INDUCED CHANGES IN LACTOSE HYDROLYSED MILKS

Doctoral Dissertation

Olli Tossavainen

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Faculty of Chemistry and Materials Sciences
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Abstract

Carbohydrate reduced lactose hydrolysed milks (CRHM) tasting the same as normal milk have become very popular in several countries. Hitherto their quality during manufacturing and storage has not been studied very closely and compared to the quality of traditional lactose hydrolysed milk (HM) or unhydrolysed milk (UM). The changes caused by the Maillard reactions and proteolysis were followed in different types of lactose hydrolysed skim milks.

Lactose hydrolysed milk is highly vulnerable to Maillard reactions and therefore its nutritional quality is often impaired due to the reactions between free amino groups of lysine and reducing sugars. The molar quantity of reducing sugars in HM is almost twofold that in UM. The early stages of Maillard reactions were followed by measuring furosine, which can be released from the Amadori products of lysine and reducing sugars. The blockage of lysine was estimated on the basis of furosine.

Pasteurization of lactose hydrolysed skim milk caused only a small increase in furosine compared to UM. In more intensive heat treatments, ESL- and UHT-treatment, furosine formation was more intensive and was found to be closely related to the level of reducing sugars in lactose hydrolysed milk. The hydrolysis products of lactose, i.e. glucose and galactose, were more reactive than lactose in the Maillard reactions occurring during UHT-treatment. As their taste is also sweeter than that of lactose, reduction of the content of monosaccharides in milk improves the nutritional quality of proteins and at the same time preserves the original taste of milk. The almost total removal of lactose with a chromatographic process reduced the blockage of lysine to a negligible level and improved the nutritional value of protein in carbohydrate free milk (CFM) during storage compared to HMs.

Proteolysis in lactose hydrolysed UHT-milks is a common problem caused by the indigenous plasmin enzyme system in milk, proteases of contaminating microbes or side activities of the lactase enzyme preparation used. The proteolytic changes and Maillard reactions occurring in lactose hydrolysed milk were largely avoided by separating lactose from milk proteins in a chromatographic process and at the same time subjecting the protein fraction to a heat treatment largely inactivating the proteolytic enzymes. UHT-milk made from fractionated milk protein (CFM) had a longer shelf life than traditionally manufactured HM.

Keywords Lactose hydrolysed milk, furosine, available lysine

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Tiivistelmä

Normaalilta maidolta maistuvat laktoosittomat maitojuomat, joiden hiilihydraattipitoisuutta on alennettu normaalista maidosta (CRHM), ovat tulleet erittäin suosituiksi viime vuosina useissa maissa. Tähän mennessä niiden laatua valmistuksen ja säilytyksen aikana ei ole tutkittu ja verrattu perinteisiin laktoosihydrolysoituihin maitoihin (HM) tai normaaliin maitoon (UM). Maillardin reaktion ja proteolyysin aiheuttamia muutoksia seurattiin erilaisissa laktoosihydrolysoiduissa rasvattomissa maidoissa.

Laktoosihydrolysoidut maidot ovat herkkiä Maillardin reaktiolle ja siksi niiden proteiinien ravitsemuksellinen laatu on usein heikentynyt lysiinin vapaiden aminoryhmien ja pelkistävien sokerien välisten reaktioiden vuoksi. Pelkistävien sokerien moolipitoisuus HM:ssa on lähes kaksi kertaa suurempi kuin UM:ssa. Maillardin reaktion alkuvaihetta seurattiin mittaamalla furosiinia, joka voidaan vapauttaa lysiinin ja pelkistävien sokerien muodostamista Amadori-tuotteista. Tuhoutuneen lysiinin osuutta arvioitiin furosiinin perusteella.

Rasvattoman hydrolysoidun maidon pastörointi aiheutti vain pienen lisäyksen furosiinipitoisuudessa verrattuna hydrolysoimattomaan rasvattomaan maitoon. Voimakkaammissa ESL- ja UHT-lämpökäsittelyissä furosiinin muodostuminen oli merkittävästi suurempaa ja se oli suhteessa pelkistävien sokerien määrään laktoosihydrolysoidussa maidossa. Laktoosin hydrolyysituotteet, glukoosi ja galaktoosi, aiheuttivat UHT-käsittelyssä voimakkaamman Maillardin reaktion kuin laktoosi. Ne ovat myös makeampia kuin laktoosi, joten monosakkaridien pitoisuuden alentaminen maidossa paransi maidon proteiinien ravintoarvoa ja samalla oli mahdollista säilyttää maidon alkuperäinen maku. Laktoosin erottaminen maidosta lähes täydellisesti kromatografisen erotuksen avulla esti lysiinin tuhoutumisen lähes täysin hiilihydraatittomassa UHT-maitojuomassa (CFM) säilytyksen aikana.

Proteolyysi on yleinen ongelma laktoosihydrolysoiduissa UHT-maidoissa, joissa sen aiheuttajana on maidon luonnollinen plasmiini-entsyymijärjestelmä, kontaminoivien mikrobien proteaasit tai lisätyn laktaasientsyymin sivuaktiivisuudet. Proteolyyttiset muutokset ja Maillardin reaktio voitiin suuressa määrin välttää erottamalla laktoosi proteiinista kromatografisen erotuksen avulla ja samanaikaisesti lämpökäsittelemällä proteiinijae siten, että suuri osa proteolyyttisistä entsyymeistä inaktivoitui. Fraktioidusta maitoproteiinista valmistettu UHT-maitojuoma (CFM) säilyi pidempään kuin perinteisesti valmistettu laktoosihydrolysoitu UHT-maito.

Asiasanat laktoosihydrolysoitu maito, furosiini, käyttökelpoinen lysiini

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Preface

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Olli Tossavainen

Contents

List of Publications:

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II. Tossavainen, O., Kallioinen, H., Effect of lactose hydrolysis on furosine formation in skim milk during pasteurisation, *Milchwissenschaft* 62 (2) (2007) 188-191.

III. Kallioinen, H., Tossavainen, O., Changes during storage of lactose hydrolysed extended shelf life (ESL) milk, *Milchwissenschaft* (accepted for publication)

IV. Tossavainen, O., Kallioinen, H., Proteolytic changes in lactose hydrolysed UHT milks during storage, *Milchwissenschaft* 62 (4) (2007) 410-415.

V. Tossavainen, O., Kallioinen, H., Effect of lactose hydrolysis on furosine and available lysine in UHT skim milk, *Milchwissenschaft* 63 (1) (2008) 22-26.

VI. Tossavainen, O., Kallioinen, H., Furosine formation and proteolytic changes in carbohydrate reduced UHT-milks, *Milchwissenschaft* (accepted for publication)

The author's contribution in the appended publications:

Publication I: Olli Tossavainen acted as a co-writer for the review article. Paul Jelen wrote about crude cell extracts and Tossavainen about the new technology used in the production of lactose free milk.

Publication II: Olli Tossavainen planned the tests, processed the results and wrote the article together with Harri Kallioinen.Test trials were performed together with pilot plant personnel.

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Publication IV: Olli Tossavainen planned the test trials in cooperation with the project team. He processed the results and wrote the manuscript.

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Other Relevant Publications:

Tossavainen, O., Sahlstein, J., Process for producing a lactose-free milk product, EP1503630 B1 (2003).

Silfverberg, P., Tossavainen, O., Jonson, V., Menetelmä vähälaktoosisten ja laktoosittomien hapanmaitotuotteiden valmistamiseksi, FI118115 B (2007).

List of Abbreviations:

α-amino-N	α-amino nitrogen
Arg	arginine
a_w	water activity
BV	biological value
CCE	crude cellular extract
CFM	carbohydrate free milk
CML	carboxymethyl lysine
CN	casein
α_{s1}-CN	α_{s1}-casein
α_{s2}-CN	α_{s2}-casein
β-CN	β-casein
γ^1-CN	γ^1-casein etc.
κ-CN	κ-casein
CRHM	carbohydrate reduced hydrolysed milk
D	decimal reduction time
Da	Daltons
FDNB	fluorodinitrobenzene
HM	hydrolysed milk
HMF	hydroxymethylfurfural
Ile	isoleucine
α-LA	α-lactalbumin
β-LG	β-lactoglobulin
LAL	lysinoalanine
LH	low heat
LSHM	low sweetness lactose hydrolysed milk
LTI	low-temperature inactivation
Lys	lysine
MRP	Maillard reaction products
N	nitrogen
NF	nanofiltration
NPU	net protein utilization
PA	plasminogen activator
t-PA	tissue type plasminogen activator
u-PA	urokinase type plasminogen activator
PAI	plasminogen activator inhibitor
PI	plasmin inhibitor
PP	proteose peptone
PP5, $PP8_{slow}$,$PP8_{fast}$	fractions of proteose peptone
SH	sulfhydryl
SMUF	simulated milk ultrafiltrate
TD	true digestibility
Trp	tryptophan
UF	ultrafiltration
UHT	ultra high temperature
UM	unhydrolysed milk

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1. Introduction

Approximately 70% of the world's population is deficient in intestinal lactase (β-D-galactosidase, IUB 3.2.1.23), the enzyme necessary to digest lactose (NDC, 1985). This condition, with a very low lactase activity in the jejunal mucosa, is called hypolactasia (Sahi, 1994a). However, the prevalence of adult-type hypolactasia varies from less than 5% to almost 100% between different populations of the world (Sahi, 1994b). Basically three types of hypolactasia are known (Dahlqvist, 1983): a) adult-type hypolactasia, which is very common in most populations (Sahi, 1994b), b) secondary lactase deficiency, in which intestinal lactase activity can be lost partly or entirely due to operation, infection, infestation, allergy, malnutrition or celiac disease (Asp, 2001; Savaiano, 2002) and c) congenital type hypolactasia, which is a very rare type with complete lactase deficiency (Mustapha et al., 1997).

It has been estimated that at least 90% of all adults on earth are lactase deficient (Dahlqvist, 1983). In the USA alone, the number of lactose intolerant individuals has been estimated to be about 50 million (Sloan, 1999). Substantial evidence supports the view that lactase activity decreases from infantile levels to adult levels (a 10-20 fold reduction) between the ages of 3 and 5 years in 75% of the world's population (Savaiano, 2002). This means that the status, called adult-type hypolactasia, is not only a problem for adults but may also limit milk consumption of children (Sahi, 1994b). The manifestation age of hypolactasia appears to vary significantly between different populations (Dahlqvist, 1983). In most populations lactase activity begins to decline soon or a few years after weaning. In particular, in populations where the prevalence of hypolactasia is high in adulthood the manifestation occurs in the majority of children at the age of (1-)2 to 7 years (Sahi, 1994b).

Adult-type hypolactasia is a genetically determined enzyme defect (Enattah et al., 2002; Kuokkanen et al., 2003). Lactase persistence resulted from a genetic mutation, which occurred thousands of years ago (Simoons, 1980; Sahi, 1994b). Both the lactase persistent and lactase non-persistent phenotypes are determined by different alleles at a single gene locus where the allele responsible for adult-type lactase persistence is dominant over that which causes lactase non-persistence (Swallow and Harvey, 1993). It was found that the ability to maintain high levels of lactase activity throughout life is strongly related to the development of dairying in certain areas of the world, such as the Middle East, Northern Europe and Northeastern Africa (Sahi, 1994a, 1994b; Simoons, 1980; Mustapha et al., 1997). People having some kind of lactase nonpersistence have difficulty in properly digesting or otherwise utilizing the nutrients of fresh milk and milk products (Holsinger and Kligerman, 1991; Mustapha et al., 1997). Nowadays several genetic tests are available to study lactase persistence in an indvidual subject.

Although wide variation exists in the results of the lactose quantity needed to cause symptoms of lactose intolerance in consumers with hypolactasia (Marteau et al., 1998, 2002; Asp, 2001), a continuous demand for low lactose or lactose free dairy products exists especially in countries with a traditionally high milk consumption and high prevalence of hypolactasia (Dahlqvist, 1983; Jelen and Tossavainen, 2003). However, the sweet taste of lactose hydrolysed milk is different from that of traditional milk and it may cause some consumers to avoid milk products (Harju, 2004; Walstra et al., 2006). Lactose hydrolysed milk often has quality defects such as browning, off-tastes, texture defects due to Maillard reactions, side activities of the lactase enzyme used or proteolytic enzymes of milk origin or from contaminating bacteria (Zadow, 1993; Mittal et al., 1991). Lactose hydrolysed dairy products have benefits, which usually overcome the problems associated with product quality. In addition to the avoidance of symptoms of lactose intolerance, hydrolysis of lactose in milk was found to enhance the absorption of calcium in lactase-deficient subjects (Birlouez-Aragon, 1988).

In Finland the incidence of hypolactasia is about 17% (Sahi, 1994b). Here the need for low lactose or lactose free products has been exceptionally high due to the high consumption of milk. During the past six years lactose free milk with the taste of normal milk has strongly gained in popularity in Finland and other countries (Jelen and Tossavainen, 2003) and has become one of the basic milks in Finland. Valio Ltd first launched the product in September 2001 based on patented technology. New lactose hydrolysed products are also being launched elsewhere (Manzi et al., 2007).

The highest residual lactose level in lactose free products accepted by the authorities in Finland is very low, 0.01%. Therefore production of lactose free products only with enzymatic hydrolysis leads to even more enhanced sweetness and increases the risk of quality defects during the storage time.

The effect of lactose hydrolysis on the Maillard reaction and the nutritional value of protein in lactose hydrolysed milk and carbohydrate reduced lactose hydrolysed milk were estimated during storage after different heat treatment processes. The changes during storage at different temperatures were monitored and alternative processes to avoid the detrimental changes in milk were compared.

1.1. Hydrolysis of lactose in milk

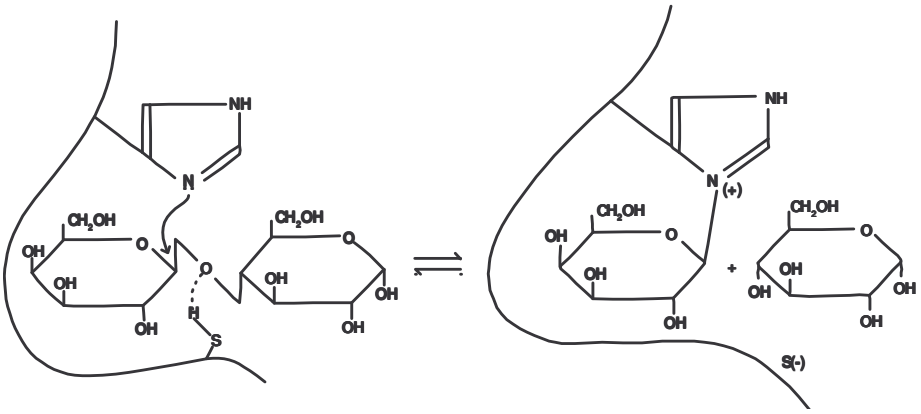
Enzymic hydrolysis of lactose can be described by three steps which allow hydrolysis to glucose and galactose as well as a galactosyl transfer reaction (Mahoney, 1997; 1998; Richmond et al., 1981):

1. Enzyme + Lactose → Enzyme-Lactose complex
2. Enzyme-Lactose → Galactosyl-Enzyme + Glucose
3. Galactosyl-Enzyme + H_2O → Galactose + Enzyme

or

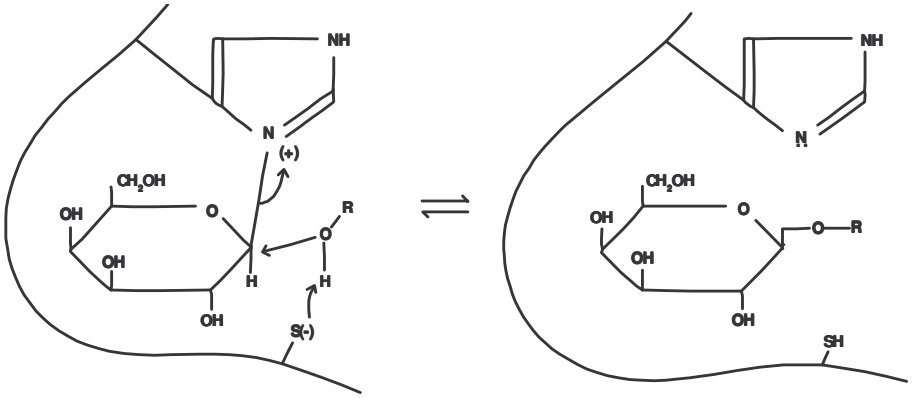
4. Galactosyl-Enzyme + acceptor sugar → Oligosaccharide + Enzyme

Figure 1 shows one proposed mechanism of hydrolysis of lactose and formation of oligosaccharides for neutral pH lactases (Mahoney, 1997; Zárate and López-Leiva, 1990; Shukla, 1975). Lactase of *Escherichia coli* is one of the most extensively studied β-galactosidases (Panesar et al., 2006) and it serves as a model for understanding the catalytic mechanism of β-D-galactosidase action. Its structure and mechanism of action were recently reviewed by Matthews (2005).



1a. A molecule of lactose on the active site of β-galactosidase.

1b. β-galactosidase complex + glucose.



1c. β-galactosidase - galactose complex plus a molecule of H_2O or mono-, di-, tri-, tetra saccharide.

1d. A molecule of galactose or di- or higher oligosaccharide on the active site of the β-galactosidase.

Figure 1. Proposed mechanism of lactose hydrolysis and formation of oligosaccharides by β-galactosidases (Zárate and López-Leiva, 1990; Shukla, 1975).

The balance between steps 3 and 4 in the reaction is usually heavily on the side of step 3. However, depending on the conditions and on the enzyme itself, wide variation occurs in this respect (Mahoney, 1998; Smart, 1993; Zárate and López-Leiva, 1990). The amount and nature of oligosaccharides formed during enzymatic hydrolysis of lactose depends strongly on the initial lactose concentration and the nature of the substrate, on the enzyme source and concentration and on the reaction time (Zárate and López-Leiva, 1990; Mahoney, 1998). Using lactose in buffer solution yields larger amounts of oligosaccharides than using milk or milk products (Zárate and López-Leiva, 1990). Forsman et al. (1979) described a simple empirical static process model for the hydrolysis of lactose with *Kluyveromyces lactis* β-galactosidase. It was applicable over wide ranges of temperature and enzyme concentration.

The kinetics of hydrolysis with soluble and immobilized β-galactosidase of *Kluyveromyces fragilis* were studied by Carrara and Rubiolo (1996). Mitchell developed a non-linear kinetic model for hydrolysis of lactose using an immobilized lactase reactor (Mitchell and Hourigan, 1993). Product inhibition of the enzymatic hydrolysis of lactose has been demonstrated by both D-glucose (non-competitive) and D-galactose (competitive inhibition) (Chen et al., 1985). Extensive hydrolysis of lactose was studied by Palomaa (2001). Properties, production and purification of β-galactosidases from different microbial origins were recently reviewed by Panesar et al. (2006). The characteristics of different microbial β-galactosidases were reviewed by Mahoney (1997).

The mechanism of enzyme action indicates that the enzyme will transfer galactose to nucleophilic acceptors containing a hydroxyl group (Figure 1). When the acceptor is water, the result is galactose, when the acceptor is another sugar the result is di-, tri- or higher saccharides, collectively called oligosaccharides. Since all of the sugars present can act as acceptors the result is a complex mixture. Complete removal of oligosaccharides can be achieved only (if at all) by prolonging the reaction time far beyond that required for hydrolysing 90% of the lactose (Jeon and Mantha, 1985; Mahoney, 1998). Among enzymes of commercial interest, the β-galactosidase from *Aspergillus niger* produces less oligosaccharides than that from *A. oryzae* and the β-galactosidase from *K. lactis* produces less than that from *K. fragilis* (Mahoney, 1997). Among the bacterial enzymes, those from *Streptococcus thermophilus* (Smart, 1993), *Bacillus circulans* (Mozaffar et al., 1984) and *Saccharopolyspora rectivirgula* (Nakao et al., 1994) produce high levels of oligosaccharides (~40%).

1.2. Manufacture of low lactose and lactose free milks

1.2.1. Hydrolysis with soluble enzymes

Lactase enzyme (β-D-galactosidase i.e. β-D-galactoside galactohydrolase, E.C. 3.2.1.23) is widely distributed in nature and can be isolated from different sources such as plants (almonds, peaches, apricots, apples), animal organs, yeast, bacteria and fungi (Richmond et al., 1981). Lactases were first proposed for dairy applications in 1950 (Van Dam et al., 1950). Lactose hydrolysed milks have been under development since the 1970s, when the first β-galactosidases became commercially available. Nowadays lactase is one of the most important enzymes used in food processing (Panesar et al., 2006).

The process is simple and does not need special equipment in dairy plants (Zadow, 1986). When using a single-use enzyme for lactose hydrolysis several factors must be considered. These include the substrate, pH of operation, maximum temperature and contact time permissible, enzyme activity and cost. An extensive contact time at 35-45°C may be required to reduce costs, but with milk this usually results in extensive microbial growth. Alternatively overnight holding at refrigeration temperature may be employed (Zadow, 1986). Soluble lactases used are usually of microbial origin (Mahoney, 1997; Greenberg and Mahoney, 1981) (Table 1).

Table 1. Properties of microbial lactases (Mahoney, 1997).

Source	Molecular weight ($x10^3$)	pH optimum[a]	Temperature operation range (°C)	Activators	Ionic inhibitors[b]
A.niger	124	3.0-4.0	55-60	none needed	none
A.oryzae	90	5.0-6.2	50-55	none needed	none
K. lactis	228	6.5-7.3	35	K, Mg, Mn	Ca, Na
K. fragilis	201	6.6	37	K, Mg, Mn	Ca, Na
E. coli	464	7.2	40	Na, K, Mg	-
B. circulans	240	6.0	60	none needed	-
B. subtilis	88	6.5-7.0	50	none needed	-
B. stearothermo-philus	116	5.8-6.4	65	Mg	-
L. acidophilus	540	6.2-6.6	55	Mg	-
S. thermophilus	464	7.1	55	Na, K, Mg	Ca

[a] dependent on strain/source
[b] ionic species likely to be found in dairy products
- = data not available

Dahlqvist et al. (1977) used minute amounts of lactase enzyme for production of lactose hydrolysed UHT-milk in a process in which lactase was sterile filtered into the package after the UHT-treatment. In the sterile product lactase had a long time for hydrolysis at ambient temperature. The enzyme quantity needed was only about 1% of the amount needed for hydrolysis under non-sterile conditions. This new method for producing lactose hydrolysed milk was patented by TetraPak (1977). Other authors have also recommended hydrolysis of lactose after the heat treatment in order to avoid progress of Maillard reactions (Mendoza et al., 2005). They also suggested limiting the degree of hydrolysis to obtain a degree of hydrolysis between 80 and 90% in order to avoid excessive sweetness. According to Walstra et al. (2006) this process has not been successful, because the product is relatively expensive and most consumers still consider the taste to be too sweet. Vasala et al. (1996) patented a method to reduce the sweetness of lactose hydrolysed UHT-milk by adding potassium salt of an organic acid, such as citrate, malate, gluconate or lactate at up to 80 mmol/l, optimally 15-45 mmol/l. Flynn et al. (1994) reduced the sweetness of lactose hydrolysed milk using potassium chloride.

Tamura et al. (1991) developed low sweetness lactose hydrolysed milk (LSHM) by treating concentrated milk with a β-galactosidase with high transgalactosylation activity. The content of oligosaccharides was about 25% of the total sugar when treating 30% reconstituted skim milk at 42°C. The content of monosaccharides was decreased from 75% in the commercial product to 50% of total sugar and this resulted in a reduction in the sweetness of lactose hydrolysed milk. Products were compared with constipated and lactose intolerant subjects. In the case of constipated subjects 300 ml of LSHM was given daily for two weeks. Their stools became softer than those of subjects not given any milk. However, no difference was observed in the hardness of the stools of subjects drinking LSHM or normal milk. Giving 180 ml of LSHM or 75% hydrolysed commercial milk per day to lactose intolerant individuals caused no difference in symptoms between the two products. In this case the daily dose was probably too small to cause any differences in symptoms.

Broome et al. (1983) found off-taste formation both with fungal and yeast lactases when used in yoghurt manufacture. Lactases were added together with the culture organisms. Dariani et al. (1982) reported off-flavours and premature coagulation in lactose hydrolysed yoghurts, which were attributed to protease contamination in the lactase preparations. Early commercial β-galactosidases contained side activities, especially proteolytic activities, which caused e.g.

off-tastes in the fresh products (Mittal et al., 1991). Enzyme quality varied from batch to batch and a strong negative correlation was observed between the proteolytic activity of the lactase preparation and the retail price of the product (Mittal et al., 1991). Higher priced lactases were more likely to contain lower levels of proteases. According to Zadow (1984) in Japan a method was developed with which it was possible to inactivate the harmful side activities with gamma radiation treatment. Mittal et al. (1991) observed that during the storage of lactose hydrolysed UHT-milk off-flavours were developed and lactose hydrolysed milk had a clearly lower consumer preference score than did the unhydrolysed milk. Milks were stored at 30°C. Off-flavours were linked to the contaminating proteolytic activities in lactase preparations. Some sedimentation was also found in lactose hydrolysed milks, commencing about 24 hours after production and increasing during storage. Silfverberg et al. (2007) patented a method to produce lactose free fermented milk products by performing the hydrolysis in two phases with an intermediate heat treatment, which destroys most of the harmful side activities.

Lactose hydrolysis makes it possible to produce dairy products for lactose intolerant people, which represent the majority in most populations (Dahlqvist, 1983). Hydrolysis of lactose increases the sweetness of the product, which in many cases provides an opportunity to lower the level of added sugar. Hydrolysis of 70% of lactose in milk increases sweetness by an amount corresponding to an addition of about 2% sucrose (Zadow, 1986). In heat treated products increase of reducing sugars causes more intensive Maillard reactions.

1.2.2. Alternatives in lactose hydrolysis

Alternatives for the use of soluble lactase were reviewed by Panesar et al. (2006), Mahoney (1997), Thompkinson et al. (1991), Harju (1987a) and Zadow (1986).

1.2.2.1. Acid hydrolysis

The use of acid in lactose hydrolysis is viable only for protein-free streams such as permeate. pH adjustment can be made by direct addition of acid or by treatment of permeate with an ion exchange resin. pH is adjusted typically to 1.2 and temperature to 150°C for a short period. The hydrolysed product is brown and requires neutralization, demineralisation and decolorisation before use. This method has not been commercially adopted to any significant extent (Zadow, 1986). Several processes based on ion exchange resins have been developed, working typically at pH 1.2 and at 90-98°C (Thompkinson et al., 1991). The browning of the neutralized reaction mixture is the greatest problem, leading to complicated colour removal steps adding processing costs and causing disposal problems. Industrially this process has been used only for pure lactose solutions.

1.2.2.2. Membrane reactors

In a membrane reactor process hydrolysis is usually carried out on a protein free stream such as UF-permeate of milk or whey. The enzyme is recovered from the reaction mixture with another UF-equipment and the permeate containing hydrolysed lactose is remixed with the milk or whey retentate (Miller and Brand, 1980). However, the complexity of the process has not made it commercially attractive (Zadow, 1986). The main problem appears to be prevention of microbial growth during continuous operation at ambient or higher temperature with non-sterile feed materials (Mahoney, 1997).

Novalin et al. (2005) described another kind of membrane reactor, a membrane-diffusion reactor, in which β-galactosidase was rejected in a hollow fibre reactor and lactose in skim milk was hydrolysed continuously (Figure 2).

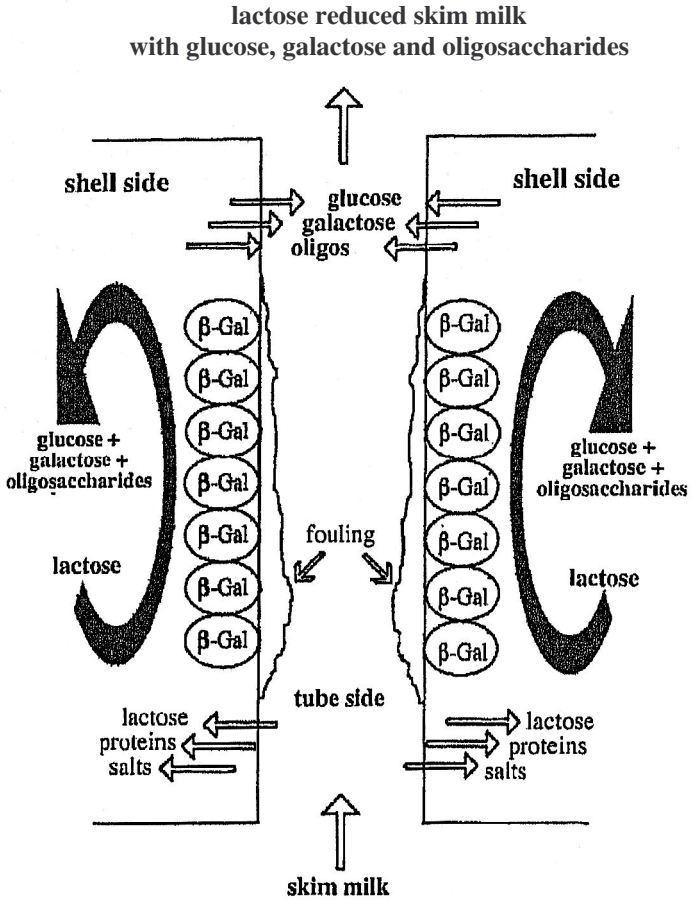


Figure 2. Principle of lactose hydrolysis by β-galactosidase (β-Gal) in a hollow-fibre module (Novalin et al., 2005).

The reactor was based on a hollow fibre module (10 kDa cut-off, 4.9 m^2 membrane area). The shell side volume was about 2.5 l and the tube side volume about 0.65 l. Feed is pumped through the tubes and enzyme is kept outside the tubes (shell side). The temperature of the feed was adjusted and enzyme solution was circulated in a closed loop. In order to control growth of the microbes in the enzyme circulation, a UV irradiation module and a sterile filtration unit were included in the circulation line. In the process a lactose conversion rate of 78.1% was achieved with a skim milk flow rate of 9.9 l h^{-1}. The authors reported that the main problems were related to the need for a high membrane area due to the low mass transfer rate, and to the control of microbial growth in the enzyme circulation. Additional studies are needed on the long-term stability and the conversion performance. The process (Figure 3) was further optimized for cost-effectiveness to work at 15$\pm$2°C with an enzyme concentration of 240 U/ml, enzyme solution flow rate of 25 l/h, and a skim milk flow rate of 9 l/h to give a productivity of 360 g $l^{-1}h^{-1}$ (Neuhaus et al., 2006).

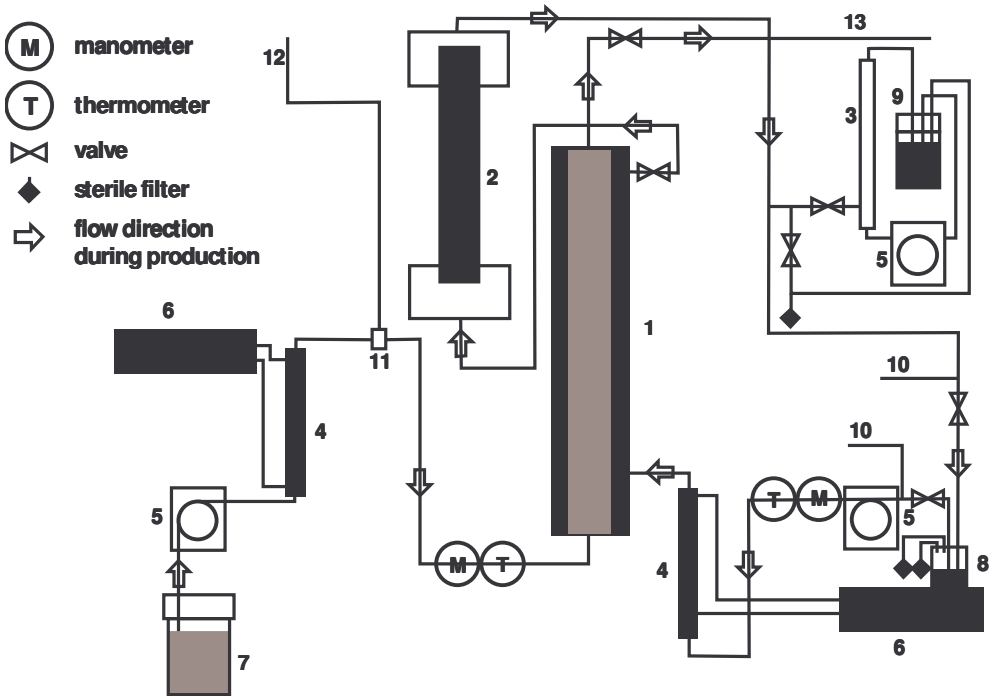


Figure 3. Scheme of a hollow-fibre reactor for lactose hydrolysis by β-galactosidase: 1. hollow-fibre module; 2. UV-module; 3. sterile filtration module; 4. heat exchanger; 5. peristaltic pump; 6. heating bath; 7. storage tank for substrate; 8. tank for enzyme solution; 9. tank for sterile filtration circulation; 10. sampling port; 11. three-way cock; 12. waste line; 13. product collection (Neuhaus et al., 2006).

Petzelbauer et al. (2002a) studied the use of two hyperthermophilic β-glycosidases from the archaebacteria *Sulfolobus solfataricus* and *Pyrococcus furiosus* in a continuous stirred-tank reactor coupled to a 10 kDa ultrafiltration module to recycle enzyme for hydrolysis of lactose at 70°C. The half lives of the enzymes were 5-7 days due to reactions with reducing sugars and adsorption onto the membrane. Using lactose as substrate it was possible to use the reactor for more than 2 weeks at a constant conversion level of 80%.

1.2.2.3. Immobilized systems

Immobilized enzyme systems appear to have great potential for large scale application for the hydrolysis of milk, permeate or whey (Zadow, 1986). Immobilized systems often utilize lactase of fungal origin (Harju, 1987a; Zadow, 1984). The carriers published include an ion exchange support (Zadow, 1986) and PVC-silica sheets, active carbon, porous glass beads, acrylic beads, cellulose triacetate or adsorption resin (Harju, 1987a). The pH-optimum of fungal lactases is about 5 but they generally retain about 50% of their activity at pH 6.8, thus making them economically feasible for hydrolysis of lactose in milk (Zadow, 1986). The low pH optimum (3.5-5.5) of fungal enzymes provides better opportunities to prevent microbial growth during processing and sanitation is also easier (Harju, 1977). The fungal enzymes are also very stable and the organisms used are on the GRAS list, which means they can be used for food purposes (Harju, 1987a). The useful lifetime of the immobilized system can be in practice several thousand hours, which significantly reduces the costs compared to those of soluble enzymes. However, if milk with a neutral pH is hydrolysed the microbial stability of the reactor becomes more difficult to control. Mahoney (1997) reviewed processing with immobilized β-galactosidase and observed that although an extremely wide variety of supports (and enzymes) employing all the primary immobilization techniques have been studied, only a few systems have been used for commercial or semi-commercial production.

Systems for processing milk use entrapped or adsorbed enzyme and systems for processing whey use adsorbed or covalently bound enzyme.

Hydrolysis of milk with immobilized β-galactosidase

The use of immobilized β-galactosidase for hydrolyzing lactose is usually targeted to reducing enzyme and processing costs. However, when hydrolysing lactose in milk a reduction of enzyme costs is much more difficult to achieve than when hydrolysing whey (Harju, 2004). However, the lactose hydrolysed milk is a much more valuable product than hydrolysed whey. In the hydrolysis of milk there are several difficulties to overcome: the neutral pH of milk encourages microbial growth except at very low or very high temperatures; milk proteins tend to adsorb onto the surface of the immobilized enzyme surface and foul the reactor; the neutral-pH β-galactosidases are not very stable when immobilized with classical techniques such as adsorption or covalent linkage (Mahoney, 1997).

Numerous β-galactosidase immobilization systems have been investigated since 1970s (Dahlqvist et al., 1973; Woychik et al., 1974; Hyrkäs et al., 1976; Harju, 1987a; Bakken et al., 1992; Thompkinson, 1993; Carrara and Rubiolo, 1996; Ateş and Mehmetoğlu, 1997), but only a few of them have been scaled up successfully and even fewer have been applied at an industrial or semi-industrial level (Panesar et al., 2006). The latest known commercial application for hydrolysis of milk was based on immobilizing neutral-pH enzyme of *K. lactis* by entrapping it in porous cellulose acetate fibres (Mahoney, 1997; Dinelli et al., 1976; Pastore et al., 1974). Immobilization gives the enzyme a shelf life of 100 days (Morisi et al., 1973). Low-lactose milk has been produced using this technology by SNAM Progetti at the Centrale de Latte in Milan (Mahoney, 1997; Panesar et al., 2006). Approximately 75% hydrolysis of lactose was achieved in a batchwise process. This process was the most effective application of immobilized β-galactosidase technology for milk in 1997 (Mahoney, 1997). This application has been developed further more recently (Cerlesi, 2000), but the main goal has not been to minimize enzyme costs but to separate the enzyme from the end product (Harju, 2004).

Hydrolysis of lactose in whey or permeate is easier because it is possible to use low-pH β-galactosidases. These processes have been reviewed comprehensively by Mahoney (1997) and Harju (1987a, 2004).

Working with thermostable neutral-pH β-galactosidases at 60°C or higher would minimize microbial growth and allow a faster catalysis. Petzelbauer et al. (1999, 2002b) studied the use of recombinant β-glycosidases from thermophilic *Sulfolobus solfataricus* and *Pyrococcus furiosus* for hydrolysis of lactose at 70°C or higher. Enzyme from *Pyrococcus furiosus* was approximately 15 times more stable than that of *Sulfolobus solfataricus*, with an operational half-life of 22 days at 80°C and pH 5.5. Immobilization of the enzyme expanded the useful pH-range by approximately 1.5 pH-units. However, the higher likelihood of Maillard browning may be a disadvantage especially if the feed is concentrated. Bakken et al. (1992) studied the use of immobilized *Bacillus circulans* lactase for hydrolysis of skim milk. At hydrolysis temperatures of 20-50°C microbial growth could not be avoided and at higher temperatures inactivation of the enzyme was significant.

Permeabilization and immobilization of microbial cells containing β-galactosidase is an interesting approach to reduce the purification costs of the enzyme and thus provide a more economic source of enzyme. This area was recently reviewed by Panesar et al. (2006). Permeabilised cells would increase the diffusion into the cell and thus enhance the use of immobilized cells. A major drawback in the use of whole cells is the poor permeability of the cell membrane to lactose. Different methods have been applied to increase the lactose permeability of microbial cells (Panesar et al., 2006). Detergents such as digitonin and cetyltrimethylammonium bromide have been successfully employed for the permeabilization

of yeast cells and the activity of permeabilized cells was many times greater than that of untreated cells. Ethanol concentration of 30-55% (v/v) caused a 100% loss of viability and up to 15-fold increase in measurable β-D-galactosidase activity during permeabilization of *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* cultures (Somkuti et al., 1998). However, other enzyme activities in the cells may cause off-tastes in the product if they cannot be inactivated.

1.2.2.4 Cellular extracts of lactobacilli

Vasiljevic and Jelen (2001) studied the use of thermophilic lactic acid bacteria *L. delbrueckii* subsp. *bulgaricus* 11842 for the production of β-galactosidases in different media. Bury and Jelen (2000) evaluated lactose hydrolysis using a disrupted dairy culture. *L. delbrueckii* subsp. *bulgaricus* 11842 was investigated for production of a crude preparation of β-galactosidase. Technical and economical feasibility were studied for producing partially lactose hydrolysed milk (DH=60%) and permeate or whey syrup. Partially hydrolysed lactose hydrolysed milk was found economically very feasible but production of whey or permeate syrup was not competitive with inexpensive sweetener commodities. During disruption of the cells other enzyme activities in addition to β-galactosidase are also released, and these may cause side reactions.

Geciova et al. (2002) reviewed different cell rupture techniques with potential for use in the dairy industry for different lactic acid bacteria. Bead milling or high pressure homogenizers (Manton-Gaulin APV or a Microfluidizer®) were found to have potential for industrial scale-up. The authors suggested that one potential application could be the use of homogenates for accelerating cheese ripening. Hydrolyzing lactose with crude homogenates affects the taste of hydrolysed milk. Vasiljevic et al. (2003) studied the sensory effects of crude cellular extracts (CCE) from *Lactobacillus delbrueckii ssp. bulgaricus* 11842 and observed that off-flavour intensity increased after 2% CCE additions. The taste of the CCE hydrolysed milk cannot be totally pure and therefore it was proposed for use with flavoured products such as yoghurt drinks (Jelen and Tossavainen, 2003). Other uses suggested were formation of disaccharides or oligosaccharides in milk due to the transferase activity of the enzyme or use as a fermentation enhancer due to the proteolytic activity.

1.2.3. Lactose free milk

In the development of lactose hydrolysed milks a new phase was reached in 2001 when Valio Ltd launched lactose free milk, which tasted like normal milk but contained <0.01% lactose (Jelen and Tossavainen, 2003; Harju, 2004). For the claim “lactose free” Finnish authorities set the limit of residual lactose content to <10 mg/100 g of product (<0.01%) and Valio has followed this rule.

Production of lactose free milk was based on a chromatographic separation process, which allows specific separation of lactose from milk (Harju, 2004; Jelen and Tossavainen, 2003). Chromatographic separation makes it possible to retain the minerals of milk with proteins, unlike in other separation techniques (Harju, 1987b, 2004). The method was patented for the separation of lactose from whey (Harju and Heikkilä, 1990) and milk (Harju, 1990). In the production of lactose free milk part of the lactose in milk was separated and the rest of the lactose was hydrolysed enzymatically, thus giving the milk its natural sweetness (Jelen and Tossavainen, 2003).

The production method was developed further on the basis of membrane techniques (Tossavainen and Sahlstein, 2003), which was an easier method to adopt in dairy plants and to

licence technology to other dairies. Milk was ultrafiltered, the UF-permeate was further nanofiltered and the permeate from nanofiltration was further concentrated with reverse osmosis. The retentate of reverse osmosis was returned back to the UF-retentate, thus returning the minerals, after which residual lactose was hydrolysed with β-galactosidase. Milk tasted the same as normal milk despite the removal of 1/3 of the lactose before the enzymatic hydrolysis.

Lange (2005) described a process for lactose free milk with no extra sweetness. The process included an ultrafiltration step in which part of the lactose was removed, and the UF-retentate was diluted to a protein concentration of 4% and lactose concentration of 3%. After this, residual lactose was hydrolysed enzymatically. The sweetness of the milk was similar to that of normal milk, but the milk had a mineral level of only 0.47%. In normal milk the mineral content is about 0.75%. This causes a difference in taste. Wang (2005) described a process for producing lactose free milk by membrane techniques. Milk was first ultrafiltered and then diafiltered. The UF-permeate was nanofiltered to give a lactose fraction (NF-retentate) and a mineral fraction (NF-permeate). The UF-retentate was then added to the NF-permeate to produce a final product having at least 95% lactose removed. This product was intended for people with lactose intolerance, diabetes mellitus or people on the Atkins' diet or a regular low-calorie diet. By removing lactose the sweetness also disappears. Therefore the product tastes different from normal milk or it must be sweetened in other ways.

Choi et al. (2007) described a process for lactose hydrolysed milk with low sweetness using nanofiltration. Raw milk was first treated with β-galactosidase at 4°C for 40 hours to hydrolyse lactose fully. During nanofiltration with a concentration factor of 1.6, part of the monosaccharides were removed through the membrane. After filtration the milk was diluted to adjust the protein content to that of normal milk. Lactose hydrolysed milk had sweetness similar to that of normal milk. Retention of crude protein, Ca, Na and riboflavin were 99, 97, 77 and 80% respectively. A benefit is a simple process, disadvantages are a need to hydrolyse all lactose in milk before nanofiltration and partial removal of monovalent ions and riboflavin.

1.3. Heat treatment processes for milk

Heat treatment destroys all or part of the microorganisms present in milk. It also inactivates some of the enzymes naturally occurring in milk. Heating methods have been developed to destroy only the pathogenic organisms in milk (pasteurization) or destroy all microorganisms and inactivate enzymes (sterilization).

The heat treatment history of milk can be analysed by analysing which enzymes have remained active. Alkaline phosphatase must be inactivated to confirm adequate pasteurization of milk. The peroxidase test should be positive to prove that the milk has not been heated too much (Kessler, 2002). An exception to this is accepted in the case of high temperature pasteurized milk, ESL- and UHT-milk and sterilized milk. Some major heat-induced changes in milk are described in Figure 4.

Pasteurization is carried out at temperatures below 100°C, at which enzyme activity is only partly destroyed and the number of microorganisms is reduced. This gives the product a shelf life of 5 to 20 days depending on the storage temperature. A shelf life of 20 days can only be achieved if the storage temperature does not exceed 5°C. ESL-treatment (extended shelf life) gives the product a shelf life which under refrigerated conditions is longer than for pasteurized milks but shorter than for ultra-high-temperature treated (UHT) milks. In ESL-technique the microbial count is reduced beyond normal pasteurization and the milk is packed under strictly hygienic conditions. Taste and properties of the product are close to those of pasteurized milk and the milk is sold from a fridge. ESL treatment is based on UHT-

technology (Rysstad and Kolstad, 2006). Conditions for different heating processes of milk are presented in Table 2.

Table 2. Conditions for different heating processes (Hinrichs and Kessler, 1995; Rysstad and Kolstad, 2006).

Process	Temperature (°C)	Time
Pasteurization		
- Batch	62-65	30-32 min
- Short time	72-75	15-30 s
- High temperature	at least 85	at least 4 s
Thermization	60-65	10-20 s
ESL-treatment	130-145	<1 s
Sterilization		
- In container	109-120	40-20 min
- UHT-treatment	135-150	20-2 s

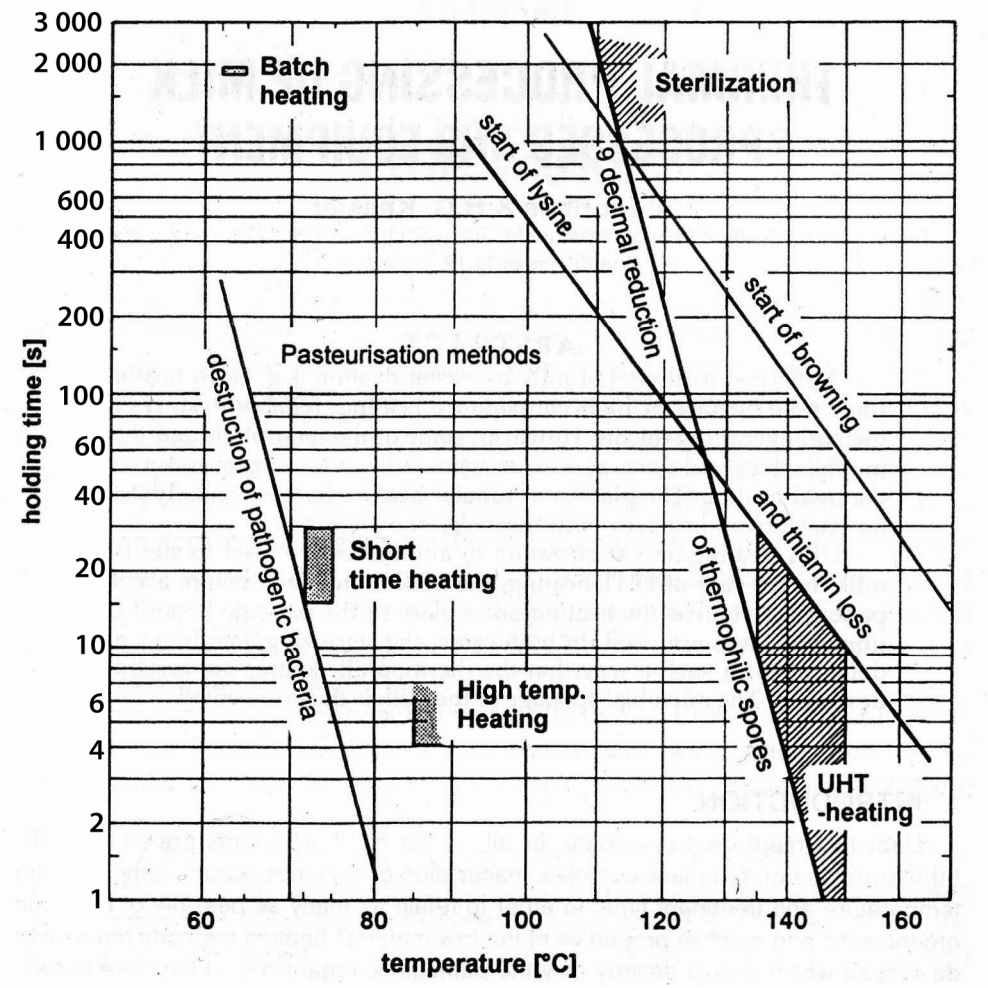


Figure 4. Temperature-time plots for some heat-induced changes occuring in milk (Kessler, 1992).

1.4. Effects of heat treatment and storage on the quality of lactose hydrolysed milk

1.4.1. Maillard reactions

According to Finot (1983) there are two chemical reactions of nutritional and physiological importance in the heating of milk. The Maillard reaction is the most important quantitatively and nutritionally, and because of this reaction the bioavailabilities of lysine, methionine, tryptophan and B-vitamins are reduced. The formation of lysinoalanine induces negligible nutritional effects, but provokes a renal defect (renal cytomegaly) in rats at level 5 times higher than the level found in some sterilized milks.

The Maillard reaction is a nonenzymatic browning reaction between reducing sugars and free amino groups (Brands and van Boekel, 2002, 2001; O'Brien, 1997; van Boekel, 2001, 1996; Ashoor and Zent, 1984; Berg and van Boekel, 1994). The reaction is very complex and its evolution depends on the reaction conditions (O'Brien and Morrissey, 1989a). In lactose hydrolysed milks the Maillard reaction is a major problem because in hydrolysis of lactose the molar quantity of reducing sugars is doubled (Evangelisti et al., 1999). Furthermore, the reactivities of glucose and galactose are higher than that of lactose (Brands et al., 2000). Galactose is more reactive than glucose (Brands et al., 2000), which is explained by the higher amount of galactose present in acyclic form, which is the form in which the sugar reacts with the lysine residues in the Maillard reaction (Hayward and Angyal, 1977). The early phases of the Maillard reaction are often monitored by furosine, which represents the Amadori products formed in reactions between free amino groups and reducing sugars and resulting in a loss of their availability (Erbersdobler and Somoza, 2007; Erbersdobler and Faist, 2001). Other compounds used as markers of nutritional quality are N(epsilon)-carboxymethyl lysine (CML), hydroxymethylfurfural (HMF), pyrraline, pentoside and pronyllysine (Erbersdobler and Somoza, 2007). These markers appear to be useful markers for the advanced stages of the Maillard reaction.

The functional group most reactive in milk proteins is the ε-amino group of lysine, but other reactive N-groups include e.g. the indolyl group of tryptophan, the imidazole group of histidine, the guanidino group of arginine and the α-amino group of proteins and free amino acids (O'Brien, 1997). More than 90% of all free amino groups in milk protein are those of the lysine side chain (Burvall et al., 1977). Table 3 lists the amino acid compositions and reactive N-containing functional groups of the major milk proteins.

Once started, the Maillard reaction continues easily to the further stages during storage (Möller et al., 1977b; Brands et al., 2002). The Maillard reaction is sensitive to water activity (a_w), occurring with maximum velocity at a_w 0.3-0.7 depending on the kind of food (Eichner, 1974). In lactose hydrolysed milk powder an available lysine loss of about 40% occurred after storage of 6 months at room temperature (Burvall et al., 1978). Figure 5 shows a simplified scheme of the Maillard reaction.

Table 3. Content of amino acids with reactive N-containing functional groups in the major milk proteins[a] (O'Brien, 1995).

	Amino acid residues				
Protein	Total	Lysine	Tryptophan	Histidine	Arginine
α_{s1}-casein	199	14	2	5	6
α_{s2}-casein	207	24	2	3	6
β-casein	209	11	2	6	5
K-casein	169	9	1	3	5
β-lactoglobulin	162	15	2	2	3
α-lactalbumin	123	12	4	3	1

[a] Excluding α-NH_2 groups; the amino acid contents may vary slightly due to genetic variation

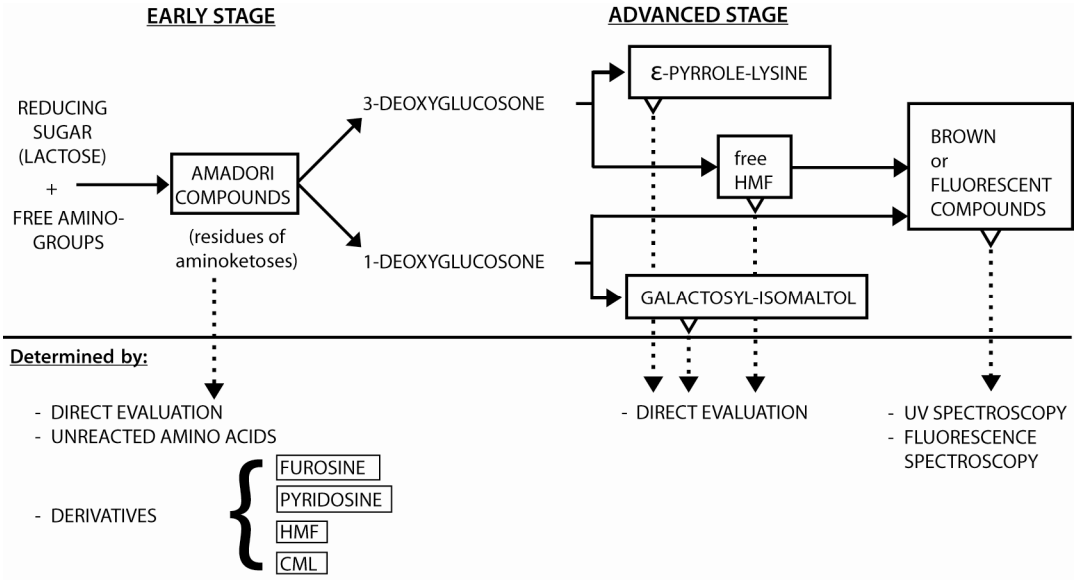


Figure 5. Simplified scheme of the Maillard reaction showing the formation of compounds (boxed) useful as indices of the severity of the heat treatment of milk (Pellegrino et al., 1995).

1.4.1.1. Nutritional quality

Heat treatment of milk is known to cause losses in vitamins of milk. UHT-treatment is reported to cause 5-20% losses in B_1, B_6, B_9, B_{12} and C-vitamins (Schaafsma, 1989). As lysine is the main amino acid in milk protein reacting in the Maillard reaction, the availability of lysine is often analysed as a marker for nutritional quality of proteins (O'Brien, 1997), although the destruction of arginine, tryptophan, cystine and histidine may also become significant depending on the processing and the storage conditions (O'Brien, 1997; Hurrell and Carpenter, 1981; Hurrell, 1990; Dworschák and Hegedüs, 1974). For example tryptophan is destroyed at 80°C more slowly than lysine, but at 100°C faster than lysine (Dworschák and Hegedüs, 1974). Moreaux and Birlouez-Aragon (1997) reported that free radicals produced during glycation of proteins could be responsible for the significant tryptophan degradation during heating of β-lactoglobulin-lactose mixture at 115°C for 6 min. Rérat et al. (2002) concluded that in milk containing low levels of blocked lysine, the nutritional loss is primarily due to loss of lysine and to a lesser extent to the decrease in the digestibility of other essential amino acids. The effects of Maillard reactions on the nutritional value of food are important especially for populations which consume a high proportion of these foods. Even if the effects

can be corrected e.g. by fortification with protein, it is worthwhile to reduce the extent of Maillard reactions (Finot, 1990).

A wide variety of methods are available for the determination of available lysine in proteins (O'Brien, 1997; Hurrell and Carpenter, 1981). Availability of lysine in milk has been studied widely (Hurrell et al., 1983; Horak and Kessler, 1981; Carpenter, 1960; Hurrell et al., 1979). Naranjo et al. (1998) found that the decrease in available lysine in casein-sugar model systems at 37°C, 50°C and 60°C followed well with first order kinetics. Lysine blocked via the Maillard reaction is unavailable for nutrition (Finot, 2005a; Sherr et al., 1989). A furosine method has been used during the past four decades to measure the amount of 'blocked' lysine particularly in milk proteins (Erbersdobler and Somoza, 2007). The advantage of the furosine method over other methods is that it directly quantifies the concentration of lactose-protein Amadori products (Erbersdobler et al., 1987; O'Brien and Morrissey, 1989a). The Maillard reactions may limit the bioavailability of undamaged amino acids by inhibiting digestive enzymes and/or by inhibiting amino acid transport at the intestinal level (O'Brien, 1997).

Birlouez-Aragon (1993) studied the effect of lactose hydrolysed milk on lens transparency in healthy young people and in elderly subjects. The author concluded that lactose hydrolysed milk is harmless to healthy young people who actively metabolise galactose and thus maintain a low plasma galactose level. In elderly subjects and patients with sugar disorders, lactose hydrolysed milk can increase the already high plasma galactose levels, thus increasing the risk of cataracts. Birlouez-Aragon et al. (1990) studied lactose intake and lactase activity in 197 persons between 55 and 90 years old, of whom 111 had advanced cataracts (88 non-diabetic, 23 diabetic) and 86 were free from cataracts (51 non-diabetic and 35 diabetic). The presence of lactase activity or a high lactose intake (more than 10 g daily) was a risk factor for senile cataract only for subjects 55 to 76 years old. For diabetic subjects consuming more milk products than non-diabetic subjects, only lactose intake appeared to be a risk factor.

The cataractogenic effect of lactose intake could be related to impaired galactose metabolism in elderly people. This was shown by the significantly higher plasma galactose values observed after 0.5 g/kg of galactose was taken by subjects more than 60 years of age than by persons of 20 to 30 years of age. In elderly diabetics, galactose values were even higher. Erythrocyte galactokinase activity was low in elderly subjects. Those with cataract had lower UDP-galactosyl transferase activities than controls. When ingesting galactose alone it will lead to high plasma levels of galactose, but when ingested with equimolar amounts of glucose there is virtually no increase in blood serum galactose (de Vrese, 1993; Zadow, 1986). Elderly people or diabetic subjects have an increased risk of galactosemia due to impaired galactose metabolism (de Vrese, 1993).

Hydrolysis of lactose has for long been known to enhance Maillard reactions during heat treatment. Dahlqvist et al. (1977) reported that lactose hydrolysed UHT-milk (0.5% fat) could be stored for 1 month at room temperature without significant loss of biologically available lysine. After 3-5 months there was a 9-13% and after 8 months 26% loss of lysine. Lactose was hydrolysed after the UHT-treatment with a sterile filtered lactase.

Burvall et al. (1977) studied the nutritional protein quality of lactose hydrolysed milk after different processes with N-balance experiments on growing rats. Enzymatic hydrolysis of lactose did not cause changes in nutritional value, but in evaporated and spray-dried lactose hydrolysed milk both biological value (BV) and net protein utilization (NPU) were significantly lowered. True digestibility (TD) was not significantly affected. Spray drying was found to cause 35-40% loss of available lysine. UHT-treated, evaporated milk without spray drying had a loss of lysine <10%. In unhydrolysed spray-dried milk no losses in available lysine or in TD, BV and NPU were observed as compared to freeze-dried unhydrolysed milk. Evaporation was performed at a low temperature of 25°C. In milk lysine is not a limiting

amino acid and therefore up to about 30% loss of this amino acid would not be expected to lower the nutritional value significantly, but if the diet includes equal amounts of milk protein and gluten, lysine becomes a limiting amino acid.

Burvall et al. (1978) studied the effect of water activity during storage on the nutritional value of lactose hydrolysed dried milk. Lactose hydrolysed milk powder was found to be much more vulnerable to losses in available lysine than normal milk powder. Water activity in powder had to be very low (a_w<0.11) to keep the Maillard browning at a low level during storage. In this study the authors also followed the protein nutritional value in lactose-hydrolysed UHT milk with N-balance tests with growing rats. During storage of 3 and 8 months at 25°C available lysine in lactose-hydrolysed fluid milk decreased by 12% and 26%, respectively.

Finot et al. (1981) used the new furosine method to estimate lysine losses in heat treated milks. They found 37% of blocked lysine in lactase treated spray-dried milk. If lactose in milk was replaced with glucose the blockage of lysine in powder was increased from 0-2% to 55%. With special mild heating conditions for spray drying it was possible to reduce the blockage to 15-20%. The authors emphasized that the furosine method is only validated for milks containing lactose as reducing sugar and therefore the level of blocked lysine in lactose hydrolysed milks is only an approximation, but at a high level of blockage the accuracy is not very important.

Hurrell and Finot (1983) compared the level of lysine blockage with the furosine method in different processed milks. There was little or no blocked lysine in freeze-dried, pasteurized or UHT-treated milk or spray dried milk powder. However, powdered special formulas such as lactose hydrolysed milks, or formulas for lactose-intolerant infants containing glucose, had levels of up to 70% of blocked lysine after spray drying. With careful control of the heat treatment this could be reduced to 15%. Significant losses in milk powders occurred during storage. At 60°C after 9 weeks the product retained its natural colour, but 40% of lysine was blocked as lactulosyl-lysine. Losses in thiamin, vitamin B_6, B_{12} and pantothenic acid were also measured due to their reaction with the advanced Maillard reaction products.

Renz-Schauen (1983) analysed changes in available lysine and HMF in lactose unhydrolysed and hydrolysed UHT-milk during storage of 12 weeks. In the beginning of the storage the level of available lysine in lactose hydrolysed milk was 10% lower than in unhydrolysed milk. During storage of three months no significant changes were observed in the unhydrolysed milk at storage temperatures of either 20°C or 30°C. Similarily, in the lactose hydrolysed UHT-milk, the content of available lysine remained relatively constant at both storage temperatures. This result is rather surprising as the content of HMF increased during storage in lactose-hydrolysed milk. This indicates that the Maillard reaction had proceeded and thus also lysine blockage should have taken place. The reason for these available lysine results may be that the analysis method used was not sufficiently sensitive.

In 1985 in Germany, lysine blockage in protein of infant formulas was limited to below 15% (analysed with the furosine method) according to German legislation (Anon., 1986). Evangelisti et al. (1994) showed that furosine formation and lysine blockage can be significantly reduced by replacing part of the lactose in infant formula with low DE-value maltodextrin.

Renner et al. (1986) studied the effect of lactose hydrolysis on some quality criteria of UHT-milk. The product contained 3.5% fat and lactose was hydrolysed in the package by sterile filtered lactase (10 mg/l), which needed 14 days to reach a degree of hydrolysis above 90%. HMF content was monitored during storage for 6 months at 4, 20, and 38°C. HMF content in lactose hydrolysed milks increased much higher at all storage temperatures than in

unhydrolysed milks in any storage temperatures. Available lysine was measured to decrease only by 14% during 6 months of storage at 38°C in lactose hydrolysed milk. A similar method for available lysine was used as in Renz-Schauen (1983).

Mittal et al. (1989) studied changes in available lysine in recombined lactose unhydrolysed or hydrolysed UHT-milk during storage of 24 weeks. The milk powder used was of medium heat quality. Enzymatic hydrolysis was performed before the UHT-treatment. Degrees of lactose hydrolysis were 0%, 60%, 80% and 98%. Available lysine was analysed with a dye-binding method. Samples were stored at 5°C and 30°C. UHT treatment caused no major loss in available lysine in recombined unhydrolysed milk and 60% hydrolysed recombined milk, but caused about 3.5% loss in recombined 80% and 98% UHT-milks. Changes in available lysine at 5°C were minimal but at 30°C they were remarkable, reaching 29.5% in 98% hydrolysed milk after 24 weeks of storage at 30°C. Losses in available lysine during the storage correlated well with the degree of hydrolysis of lactose in milks.

Evangelisti et al. (1999) studied deterioration of the protein fraction by Maillard reactions in dietetic milks including lactose hydrolysed milks. Furosine method was used to estimate the blockage of lysine. Samples were stored at 4°C or 20°C for less than 5 days or for 3 months. During storage of 3 months at 20°C the level of blocked lysine increased in two lactose hydrolysed milks from 4.2% to 12.6% and from 6.5% to 17.4%. When stored at 4°C for 3 months the level of blocked lysine in the milks increased only from 4.2% to 4.8% and from 6.5% to 7.1% respectively. The authors stated that storage at $\leq$4°C should be compulsory in order to limit the protein damage that is an unavoidable consequence of the high content of reducing carbohydrates. They emphasized that the Maillard reaction may adversely influence the digestibility of the protein fraction due to steric hindrance as reported earlier by Möller et al. (1977a).

Marconi et al. (2002) and Messia et al. (2007) analysed different markers such as furosine, lactulose, fructose, and lactose from different commercial lactose hydrolysed milks in order to characterize the hydrolysis process and assess the quality of lactose hydrolysed milk.

Mendoza et al. (2005) analysed chemical indicators of heat treatment from commercial fortified and special milks. They found tagatose and fructose in lactose-hydrolysed UHT-milks, indicating that hydrolysis of lactose had been accomplished before the UHT-treatment. They showed that formation of furosine is more significant in lactose hydrolysed than in unhydrolysed milk when heated at 135, 140 or 150°C. The authors recommended that in order to avoid excessive formation of furosine, loss of lysine and changes in organoleptic characteristics, it is advisable to hydrolyse lactose under aseptic conditions after the heat treatment process.

1.4.1.2. Furosine

Acid hydrolysis of the protein-bound Amadori derivative of lactose, ε-N-(deoxy-1-D-lactulosyl)-L-lysine, is hydrolysed to 40% lysine and 32% furosine (O'Brien, 1997; Finot et al., 1981). Measurement of furosine in acid hydrolysates has become widely used to measure the amount of blocked lysine in food proteins, particularly milk proteins (O'Brien, 1995). Formation of furosine at 100-120°C can be described with apparent zero order reactions (De Rafael et al., 1997).

A possible criticism of the method is that the furosine method does not detect later products of the Maillard reaction (e.g. 5-hydroxymethyl-2-furaldehyde, melanoidins), a factor which may lead to underestimation of lysine destruction (O'Brien, 1997). However, the stability of the lactose-protein Amadori adduct is such that it would be expected to be the major or only

product of Maillard reactions in most milk systems (O'Brien, 1997). As most of the technologically advanced heat treatments of milk often restrict the Maillard reaction to its early stages (Finot et al., 1981; Erbersdobler and Dehn-Müller, 1989), measuring furosine levels makes it possible to calculate the total amount of "blocked" lysine and estimate the extent of protein deterioration (Finot and Mauron, 1972; Finot et al., 1981; Erbersdobler and Dehn-Müller, 1989; Erbersdobler and Hupe, 1991).

Finot et al. (1981) also used furosine method for lactose hydrolysed milks and found it to be suitable, but emphasized that the furosine yield in acid hydrolysis of the galactose-lysine Amadori compound may be different from that of the lactose-lysine compound. Later, the furosine method has been used for example by Evangelisti et al. (1994, 1999) and Mendoza et al. (2005).

Hurrell et al. (1983) compared nine different chemical and microbiological methods to determine lysine in milk powders during storage at 60 and 70°C. The different methods gave widely dissimilar results. The direct fluorodinitrobenzene (FDNB) technique and reactive lysine from furosine were considered to be the most reliable methods. The FDNB-difference, dye-binding lysine, *Tetrahymena* and *Pediococcus* methods all seriously underestimated reactive or available lysine in heat-damaged milk powders. *Tetrahymena* and *Pediococcus* appeared to utilize lactulosyl lysine as a source of lysine.

Originally, the furosine method was insensitive, but later it was developed to be more sensitive (Erbersdobler et al., 1987; Resmini et al., 1990), making it a sensitive indicator of lysine blockage in heated milks. Figure 5 (p. 27) shows a simplified diagram of the Maillard reaction phases in milk. Later Henle et al. (1991) developed a direct analysis method for the Amadori-product lactuloselysine. In this method the acid hydrolysis phase of the furosine method is replaced with enzymatic hydrolysis. The method gives more than 3-4 times higher values for modified lysine in milk than the furosine method. However, use of the method of Henle et al. has not been adopted widely thus far. Neither the furosine method nor Henle's method takes into account advanced Maillard reaction products formed during the heat treatment and they therefore possibly lead to underestimation of the true deficiency (Leclère and Birlouez-Aragon, 2001). Birlouez-Aragon et al. (1998) therefore proposed the use of a rapid and sensitive fluorimetric method to evaluate the heat treatment of milk. The method is based on the simultaneous determination of protein denaturation by Trp fluorecence (λ_{exc} 290nm, λ_{em} 340 nm) and formation of fluorescent advanced Maillard products (λ_{exc} 350nm, λ_{em} 440 nm) in the milk fraction soluble at pH 4.6.

Another modification of the furosine method involves enzymatic digestion and dialysis of milk proteins prior to acid hydrolysis and furosine determination, to distinguish between enzymatically available and chemically available lysine (Desrosiers et al., 1989). The authors reported that of the original lysine present in whey protein concentrate at a_w 0.97 and heated at 121°C for 83.3 min, 93% was chemically available, whereas only 76% was available enzymatically. The difference was proposed to be due to conformational changes and cross-linking, which would have limited the enzymatic digestion without necessarily destroying lysine.

Claeys et al. (2003) studied the formation of furosine in heated milks with different fat contents. Significant differences were observed between the kinetic parameters of furosine formation in whole, semi-skimmed and skimmed milk. Formation kinetics of HMF and lactulose were not affected by milk fat content. The highest reaction rate constant for furosine formation at 110°C was measured for whole milk, then semi-skimmed milk and the lowest for skimmed milk.

1.4.1.3. Colour formation

Milk colour measurements have been used to monitor advanced stages of the Maillard reaction (Rufián-Henares et al., 2002, 2004; Morales and van Boekel, 1998; Pagliarini et al. (1990). Pagliarini et al. (1990) found that the colour change (ΔE) during heating of skim milk at a constant temperature between 90 and130°C followed zero order kinetics with an activation energy of 101.8 KJ/mol. Other markers used for advanced Maillard reaction are e.g. hydroxymethylfurfural (HMF) (O'Brien and Morrissey, 1989a; O'Brien, 1997; Dehn-Müller et al., 1989), carboxymethyl lysine (CML), pyrraline and pentosidine (Erbersdobler and Somoza, 2007).

1.4.1.4. Other effects

Furniss et al. (1989) studied the effect of Maillard reaction products on zinc metabolism in the rat. They observed that casein-glucose Maillard reaction products (MRP) induced up to 6-fold increase in the quantity of Zn excreted in the urine. Similar levels of casein-lactose MRP increased urinary Zn loss 2-fold. The authors concluded that although urinary Zn excretion can be increased by the presence of MRP in the diet, this is only a minor excretory pathway and would have little influence on overall Zn nutrition in individuals fed on a diet adequate in Zn. Rehner and Walter (1991) studied bioavailability of iron, copper and zinc in suckling rats. The trace elements were given together with several isolated Maillard products and with LAL. The isolated substances had effects on bioavailability of all elements tested either on pre-resorptive or on the post-resorptive level.

Brands et al. (2000) examined the mutagenicity of heated sugar-casein systems by the Ames test using *Salmonella typhimurium* TA100. Mutagenicity could be fully ascribed to Maillard reaction products and strongly varied with the kind of sugar. Glucose and galactose showed a higher mutagenic activity, corresponding to a higher Maillard reactivity. Disaccharides showed no mutagenic activity (lactose) or lower mutagenic activity (lactulose) than their corresponding monosaccharides. Ketose sugars (fructose and tagatose) showed a remarkably higher mutagenicity compared with their aldose isomers (glucose and galactose), which was due to a difference in reaction mechanism. However, mutagenic activity of the sugar-casein systems was weak compared to chemical mutagens such as 4-nitroquinoline-N-oxide. Other Maillard reaction products have also exhibited mutagenic activity (O'Brien and Morrissey, 1989b). By contrast, proteins are well known for their antimutagenic activity (Vis et al., 1998; Brands et al., 2000) and it is possible that sugar-protein systems show no mutagenicity at all.

The tertiary structure of proteins conjugated with lactose sometimes changes as a consequence of the Maillard reaction. The complexes formed during this reaction can be more allergenic than the native protein (Kaminogawa and Totsuka, 2003). When skin reactions to β-lactoglobulin isolated from pasteurized milk were tested, its allergenicity was found to be about 100 times higher than that of unheated β-lactoglobulin (Bleumink and Young, 1968).

Lactose and its hydrolysis products glucose and galactose are precursors of aroma compounds formed non-enzymatically. Two separate types of reaction occur: one involves breakdown during heating, whereas the other involves interaction with amino acids and other nitrogenous compounds in the Maillard reaction (Calvo and de la Hoz, 1992; Izzo and Ho, 1992). The volatile compounds formed via the non-enzymatic browning reaction can be classified into three groups (Nursten, 1981):

1. simple sugar dehydration fragmentation products, e.g. furans, pyrones, cyclopentenes, carbonyl compounds and acids.
2. simple amino acid degradation products e.g. aldehydes and sulphur compounds.

3. volatiles produced by further interactions e.g. pyrroles, pyridines, imidazoles and pyrazines.

Andersson and Öste (1995) reviewed factors that affect the taste of UHT milk. Perkins et al. (2005) reported a strong correlation between concentrations of methyl ketones measured with head space analysis and various heat indices furosine, lactulose and undenatured whey proteins in UHT-milk samples containing 3.5-4% fat and stored at room temperature for 16 weeks. Valero et al. (2001) studied flavour and volatile components during storage of 90 days in whole and skimmed UHT-milk. Non casein nitrogen (NCN) increased during the storage. The increase was greater in skimmed milk samples. The main change in volatile components was increase of methyl ketones. These could be related to both proteolysis and Maillard reactions.

1.4.2. Enzymatic changes

Another problem with lactose hydrolysed milks is proteolytic and other enzymatic changes during storage (Mittal et al., 1991; DSM, 2007). This problem is also well known with lactose unhydrolysed UHT-milks with a shelf life of several months (Driessen, 1989; Leitner et al., 2006). In the UHT-process not all proteolytic or other enzymatic activities of indigenous or microbial origin are inactivated and they can cause quality defects in the product especially when stored at room temperature (Newstead et al., 2006).

1.4.2.1. The plasmin enzyme system in milk

Milk is known to contain several indigenous proteinases, the most important of which is the heat-stable alkaline serine proteinase plasmin (fibrinolysin; EC 3.4.21.7) (Fox and Kelly, 2006; Kelly et al., 2006; Swaisgood, 1995). Milk contains the complete plasmin system: plasmin, plasminogen, plasminogen activators (PAs), and inhibitors of PAs (PAIs), and of plasmin (PI). The system enters milk from blood, and plasmin activity increases during mastic infection and in late lactation. In milk plasminogen, plasmin and PAs are associated with the casein micelles and are concentrated in rennet-coagulated cheese curds and casein. The inhibitors of PAs and plasmin are soluble in the milk serum. Plasmin is a well-characterised proteinase, as are the various components of the plasmin system (Kelly and McSweeney, 2003). Bovine plasminogen is a single-chain glycoprotein containing 786 amino acid residues, with a calculated molecular mass of 88,092 Da. Plasminogen is converted to plasmin by cleavage of the Arg_{557}-Ile_{558} bond by specific proteinases, of which there are two types, urokinase- and tissue-type PAs. Plasmin is optimally active at pH 7.5 and 37°C. It is rather heat stable and partially survives UHT-processing.

Plasmin has a high specificity for peptide bonds containing Lys or Arg at the N-terminal side (Kelly and McSweeney, 2003). It has little or no activity against κ-CN, β-LG or α-LA. In fact denatured β-LG is an inhibitor (Grufferty and Fox, 1986). The principal substrate in milk is β-CN, from which it produces γ^1- (β-CN f29-209), γ^2- (β-CN f106-209) and γ^3- (β-CN f108-209) CNs and proteose peptones PP5 (β-CN f1-105/107), $PP8_{slow}$ (β-Cn f29-105/107) and $PP8_{fast}$ (β-CN f1-29) (Fox and Kelly, 2006). The specificity of plasmin for α_{s1}-, α_{s2}- and β-CNs in solution has been determined (Kelly and McSweeney, 2003). Another proteinase in milk is somatic cell-derived cathepsin D. Lysosomal elastase and cathepsin B proteases are also almost certainly present in milk (Kelly et al., 2006).

Due to its good heat stability the plasmin system causes quality problems in UHT-products with a long shelf life and when stored at ambient temperature (Enright et al., 1999; Enright and Kelly, 1999; López-Fandiño et al., 1993). Therefore much research has been conducted to inactivate the plasmin system in UHT-products. Newstead et al. (2006) reported that by

increasing the preheating treatment of the UHT-process to 90°C for 30 or 60 s and using UHT of 140 °C for 4 s, it was possible to inactivate the plasmin system so well that the milk made from reconstituted LH milk powder showed only very minor sedimentation during storage of 8 moths at 20°C and 30°C. However, such a high heat treatment of lactose hydrolysed milk easily causes severe browning by the Maillard reaction.

In heat inactivation studies of milk proteinases there is some inconsistency in the results, which is partly due to the different analysis methods used (Kelly et al., 2006). Rollema and Poll (1986) studied the kinetics and mechanism of heat-inactivation of the plasmin system in skim milk and in suspensions of casein micelles in simulated milk ultrafiltrate (SMUF). They found that heat-inactivation of both plasmin and plasminogen follows first order kinetics in the temperature range 70-140°C. In all cases the inactivation of plasmin lags behind that of plasminogen. In a suspension of casein micelles in SMUF, i.e. in the absence of whey proteins, lower rates of inactivation were observed. It was found that the kinetics of inactivation are strongly influenced by the presence of β-lactoglobulin, to a lesser extent by BSA and insignificantly by α-lactalbumin. In this effect the interaction of plasmin and plasminogen with the reactive SH-group of β-lactoglobulin plays an important role. The authors found a decimal reduction time D of 1.5 min at 85°C and 6 s at 140°C for the inactivation of plasmin and plasminogen in skim milk (Rollema and Poll, 1986).

Metwalli et al. (1998) also observed the stabilizing effect of casein and the destabilizing effect of free SH-groups on plasmin during heating. Driessen (1989) found inactivation of plasmin to follow first order kinetics and reported a D-value of 6.4 min for the inactivation of plasmin at 85°C in milk. Saint Denis et al. (2001) obtained inactivation kinetics of plasmin and plasminogen which were in line with earlier reported values, but plasminogen activators were surprisingly found to be as sensitive as plasmin and plasminogen in a milk system containing proteins with free SH-groups. At 85°C they found D-values of 126 s, 116 s and 129 s for plasmin, plasminogen and plasminogen activator respectively. The presence of β-lactoglobulin was very significant for the inactivation of both plasmin and plasmin activators. The rate of plasmin inactivation decreased in long heat treatments, probably due to the disappearance of available β-lactoglobulin for S-S linking.

Subclinical mastitis in cows has been found to increase the plasmin activity in milk ~2 fold compared to uninfected quarter (Leitner et al., 2006). Plasminogen in milk is denatured in the temperature range 50-61°C, indicating that in pasteurized milk plasminogen is in denatured form. Denatured plasminogen is more susceptible to activation by plasminogen activator (Burbrink and Hayes, 2006). Pasteurization of milk inactivates inhibitors of plasminogen activators, resulting in a net increase in plasmin activity in pasteurized milk (Prado et al., 2006). Plasminogen activators t-PA and u-PA are thermally stable in milk heated at below 75°C. Almost half of the t-PA activity and 30% of u-PA activity is lost after heating milk at 85°C for 30 s (Prado et al., 2007). Datta and Deeth (2001) reported that heat treatment of milk alters the natural balance between the activators and inhibitors in favour of the activators. This can lead to enhanced proteolysis in heated milk.

An indirect UHT-process is usually found to inactivate the plasmin system more completely than a direct UHT-process. On the other hand more whey protein denaturation, lactulose and furosine formation is found in indirectly treated milks (Elliott et al., 2005, 2003; Datta et al., 2002; Van Renterghem and De Block, 1996; Nangpal et al., 1990; Blanck et al., 1980). Snoeren et al. (1979) showed that direct UHT-treatment of 142°C, 4 s was insufficient to inactivate both the bacterial and native milk protease in whole milk. In good quality milk β-CN survived only less than 25 days when stored at 28°C, indicating the effect of milk protease. However, in an indirect UHT-process with 142°C, 4 s heat treatment, β-CN in good quality whole milk survived for 250 days, indicating inactivation of milk protease (Snoeren

and Both, 1981). Topçu et al. (2006) reported that quality defects caused by inferior raw milk were reduced by higher UHT treatment temperatures.

1.4.2.2 Microbial proteases

Enzymes from contaminating microbes in milk cause problems in the quality of UHT-milk (Chen et al., 2003; Driessen, 1989; Nieuwenhuijse, 1995; Miranda and Gripon, 1986). Particularly certain psychrotrophic bacteria in raw milk produce very heat stable proteinases (Stepaniak and Sørhaug, 1995; Collins et al., 1993; Driessen, 1989; Guamis et al., 1987; Cousin, 1982). Basically two types of thermostable enzymes can be distinguished (Stepaniak and Sørhaug, 1995): enzymes from thermophilic spore-forming bacteria or moulds and yeasts, and proteinases, lipases and phospholipase C from psychrotrophic bacteria, mostly pseudomonads. These three different enzymes may act synergistically in damaging the fat globule membrane.

Among the psychrotrophic Gram-negative bacteria, representatives of the genus *Pseudomonas* appear to be the most proteolytic organisms. Proteolytic and lipolytic enzymes of certain *Pseudomonas* strains are very heat stable and survive UHT-processes (Driessen, 1989). For proteinases of *Pseudomonas* spp. in milk, D-values of >240 min and 0.88-3.2 min at 70°C and 140°C, respectively, have been found (Stepaniak and Sørhaug, 1995). Intracellular enzymes are considered to be less harmful than extracellular because of their lower heat stability and because they contribute little to the total enzyme activity (Stepaniak and Sørhaug, 1995, Sørhaug and Stepaniak, 1991). Datta and Deeth (2003) described a method to analyse the origin of the proteinases causing proteolysis in UHT-milk. McKellar (1981) found that extracellular crude proteinases of three different strains of *Pseudomonas fluorescens* produced off-flavours during storage in UHT-milk and that the intensity of off-flavours grew in proportion to the progress of proteolysis. Off-flavours were described as bitter or astringent. UHT-milk was found to be approximately twice as sensitive to the proteolytic action of the added crude enzyme extract as pasteurized milk.

Heat stable enzymes are not easily inactivated in direct UHT-treatment, but with a combination of UHT and subsequent low-temperature inactivation treatment (LTI) many of these thermostable enzymes can be inactivated (Stepaniak and Sørhaug, 1995). LTI heat treatment takes place at temperatures of 55-70°C and lasts at least for several minutes.

1.4.2.3. Other enzymes

Lipases of microbial origin can survive UHT-treatment and cause off-flavours in milk or milk powder (Chen et al., 2003). Another source of contaminating enzymes in lactose hydrolysed milk is added β-galactosidase preparation. Mittal et al. (1991) observed that commercial lactase preparations contain proteolytic side activities, which caused off-tastes in recombined lactose hydrolysed UHT-milk during storage of 12 weeks at 30°C. The products were inferior when compared to unhydrolysed reconstituted UHT-milk or UHT whole milk. The authors compared six commercial lactases and found that the price of the enzyme and the relative amount of proteolytic side activities were inversely correlated. The hydrolysis was performed in milk at 55°C for 5 hours after recombination of the medium heat milk powder and prior to the direct UHT-treatment at 140°C for 3 s with an Alfa-Laval VTIS/VTS pilot UHT-equipment.

Harju (2004) reported that yeast lactase may cause taste defects in lactose hydrolysed milk. DSM (2007) found that one contaminating enzyme in commercial lactases causing off-tastes in milk is aryl sulfatase, which releases p-cresol from sulphates in milk. National Enzyme

Company (Anon., 2007) announced in a technical product sheet that a fungal lactase used as a digesting aid contains α-L-arabinase, glucanase, glucosidase, transferase, α-amylase and various proteolytic activities as side activities.

1.4.3. Other quality problems in lactose hydrolysed milk

Age gelation of UHT-milk during storage at ambient temperatures has been a well-known problem for a long time (Datta and Deeth, 2001; Samel et al., 1971; Zadow and Birtwistle, 1973) and also in lactose hydrolysed UHT-milk (Kocak and Zadow, 1982). Manji et al. (1986) compared age gelation in directly and indirectly UHT treated milks. Only in the direct UHT process, where plasmin and plasminogen survived better than in the indirect process, was age gelation observed. However, no relationship was found between gelation time and degree of proteolysis.

Several different mechanisms have been suggested for age gelation in UHT-milk (Nieuwenhuijse and van Boekel, 2003; Datta and Deeth, 2001). Kocak and Zadow (1985a) studied the effect of low-temperature inactivation (LTI) treatment at 55°C on age gelation of UHT whole milk. Treatment at 55°C for 60 min led to a high level of inhibition of proteolysis and the shelf life of the milk was approximately doubled. Age-gelation was measured as an increase in the apparent viscosity of milk but was not found to be related clearly to the degree of inhibition of proteolysis. Kocak and Zadow (1989) also studied the effect of LTI on age-gelation of lactose hydrolysed whole milk. The results were similar to those of unhydrolysed whole milk. LTI treatment of 40-60 minutes at 55°C after the UHT-process resulted in approximately doubling of the useful shelf life of the milk from about 50 days to about 100 days.

1.4.4. Prevention of Maillard reactions

Several approaches are used to prevent or minimize browning and the consequent antinutritional and toxicological manifestations. Sulphur-containing amino acids such as cysteine and N-acetylcysteine, and the tripeptide gluthatione can affect both enzymatic and non-enzymatic browning (Friedman and Molnar-Perl, 1990; Friedman, 1994, 1996). The antioxidant and antitoxic effects are due to a multiplicity of mechanisms including their ability to act as a) reducing agents, b) scavengers of reactive oxygen (free radical species), c) destroyers of fatty acid hydroperoxides, d) strong nucleophiles that can trap electrophilic compounds and intermediates, e) precursors for intracellular reduced gluthathione and f) inducers of cellular detoxification (Davis and Snyderwine, 1995; Friedman, 1996, 1994; Kroh et al., 1989). According to Friedman (1996), SH-containing compounds may be as effective as sodium sulphite in preventing both enzymatic and non-enzymatic browning.

Modifications of amino groups prevent them from participating in browning reactions. Treatment of foods with the enzyme transglutaminase will transform lysine amino groups to amide groups, which will effectively inhibit browning (Friedman, 1996; Friedman and Finot, 1990).

Watanabe et al. (1990) discovered that an extract from soil microorganisms catalysed the deglycation of α- and ε-fructosyl lysines to lysine. This finding suggests that these purified enzymes could be used to prevent Maillard reactions in foods and *in vivo* provided that they are safe in other respects (Friedman, 1996). Gerhardinger et al. (1995) described deglycation of Amadori compounds by a bacterial enzyme, fructosyl-N-alkyl oxidase (EC 1.5.3.), isolated from a *Pseudomonas* strain.

Schamberger and Labuza (2007) found that flavonoids of green tea can be used to control the Maillard browning during thermal processing of UHT-milk. Epicatechin and epigallocatechin gallate reduced Maillard fluorescence at the 0.1 mmol/l level, and fluorescence was negligible with added flavonoids at 1.0 mmol/l. When added to milk they reduced the production of Maillard associated fluorescence and colour changes during UHT-processing.

1.5. Aims of the present work

Carbohydrate reduced lactose hydrolysed milks (CRHM) with the natural taste of normal milk have become enormously popular in several countries. Hitherto their quality during manufacturing and storage has not been studied very closely or compared to the quality of traditional lactose hydrolysed (HM) or unhydrolysed milks (UM). The Maillard reaction and proteolysis during storage have not been previously studied at the same time in lactose hydrolysed milks.

In the literature, lactose hydrolysed milk is often described as highly vulnerable to Maillard reactions, which cause deterioration of the nutritional value of milk. The problem is apparent particularly in lactose hydrolysed milk powders and lactose hydrolysed UHT-milks, limiting their practical shelf life. For unhydrolysed UHT-milk, storage time at room temperature can be 8-9 months, whereas in lactose hydrolysed UHT-milk it is often only 3 months. A long shelf life for unhydrolysed UHT-milk can be achieved with an intensified preheating treatment, which inactivates the plasmin system and other enzymes, but this is not acceptable for lactose hydrolysed milk due to enhanced Maillard browning.

Hydrolysis of lactose in UHT-milk can be performed either before or after the heat treatment. Commercial lactases often contain proteolytic side activities, which cause proteolysis and shorten the shelf life of the product. This often leads manufacturers to perform hydrolysis before the heat treatment, which can lead to enhanced browning and lower nutritional quality. In Finland in lactose free milks the residual lactose limit is 100 mg/kg, which means that a high degree of hydrolysis is needed. The Maillard reaction is easily enhanced and nutritional protein quality deteriorates. A high dosage of enzyme is needed and the quality of the enzyme preparation becomes more crucial.

The aim of this study was to follow Maillard reactions and proteolysis in traditional HMs and in CRHMs with the taste of normal milk and to compare them with UM. The Maillard reactions were followed by furosine and the level of available lysine was estimated. Proteolysis was followed by α-amino-N, tyrosine equivalent and SDS-PAGE analyses. Changes in pasteurized, ESL- and UHT-heat treated milks were monitored. UHT-milk samples stored at different temperatures were compared to UM and traditional lactose hydrolysed UHT-milks.

Alternative processes to avoid the Maillard reaction and proteolysis in UHT-milks were compared with the aim of achieving a good shelf life for the product. Two methods to reduce carbohydrate content in milk were studied: chromatographic separation and a method based on membrane techniques.

2. Materials and methods

The materials and methods used in this study are described in more detail in publications II-VI and only a brief summary is given below.

2.1. Raw materials

2.1.1. Milk

In study II the milk used was fresh raw milk from Viikki University farm, delivered as cooled to below 5°C. In study III milk was normal fresh milk received at Valio Jyväskylä dairy. Milk was skimmed and pasteurized (72±2°C, 15 s) before further processing. In studies IV-VI the milk was normal skim milk received at Valio UHT-plant, Turenki. The skimmed raw milk was pasteurized (72±2°C, 15 s) before delivery to the Turenki plant. Commercial milk samples were collected from retail shops in the EU area and stored frozen before analyses.

2.1.2. Enzyme

Godo YNL2 lactase (Godo Shusei Co., Japan) from *Kluyveromyces lactis* was used for hydrolysis of lactose in test milks. The dosages in different studies are shown in Table 4. The activity of Godo YNL2 lactase was 6810 μmol g^{-1} min^{-1} (114 μkat g^{-1}). The degree of hydrolysis was about 98% in test milks.

Table 4. Dosage of Godo YNL2 -lactase enzyme used in different studies.

Publication	Hydrolysis stage	Dosage (%)	Dosage (% of lactose)	Dosage (U/l) and (μkat/l)
II	Prehydrolysis	0.035	0.8	2380 (40)
III	Prehydrolysis	0.09	2.0	6130 (103)
	Prehydrolysis	0.06	2.0	4090 (68)
IV	Prehydrolysis	0.09	2.0	6130 (103)
	Posthydrolysis	0.003	0.07	200 (3)
V	Prehydrolysis	0.09	2.0	6130 (103)
	Posthydrolysis	0.003	0.07	200 (3)
VI	Prehydrolysis	0.08	1.9	5450 (91)
	Prehydrolysis	0.06	1.9	4100 (69)
	Prehydrolysis	0.003	1.9	230 (4)

2.2. Manufacture of test milks

Pasteurized milks (Publication II), Figure 6
Lactose in milk was hydrolysed at 5-10°C for 24 hours, after which milk fat was separated at 50±2°C. Milk was heat treated for 15 s at temperatures between 60 and 90°C using a pilot plate heat exchanger pasteurization equipment (Otto Rüchti, Switzerland). Immediately after pasteurization the milk was cooled to below 10°C. Samples were packed into glass bottles and stored at 5°C for 8 days in a dark room.

ESL-milks (Publication III), Figure 6
Three test skim milks were produced: unhydrolysed milk (UM), hydrolysed milk (HM) and carbohydrate reduced and hydrolysed milk (CRHM). Reduction of the carbohydrate content of milk was performed according to Tossavainen and Sahlstein (2003) by ultrafiltration.

Ultrafiltration was carried out at 50±2°C and the UF-retentate was then pasteurized (80±2°C, 15 s). The ultrafiltration membrane used was GR61PP with a cut-off value of 20 kDa (DSS, Denmark). The residual lactose was hydrolysed by lactase. The minerals lost in ultrafiltration were returned as mineral powder (Valio Ltd., Finland) and the original protein content was restored by diluting with water. Lactose hydrolysis was carried out at 5-10°C for 24 h.

All the test milks were heat treated in a production scale steam infusion ESL-plant (APV, Denmark). Milk was first heated to 75±2°C for 22 s in a plate heat exchanger and then by steam infusion to 132±2°C. The heating and holding time was 1 second in all. In steam infusion added water was evaporated in a flash chamber and the temperature was simultaneously decreased to 74±2°C in 1 second. Next the milk was first homogenised and then cooled to 4°C in a plate heat exchanger. Total delay-time at 74°C was 60 s, followed by a cooling time of 30 s. Test milks were filled into 1 l carton packages and stored at 8±1°C for 8 weeks in a dark room. The ESL heat treatment of all milks was identical.

UHT-milks (Publications IV, V and shelf life study), Figure 6
Milk was normal skim milk received at the Valio UHT-plant, Turenki, Finland. The same milk batch was used both for the unhydrolysed and posthydrolysed milk tests. Very similar milk was used for the prehydrolysed milk test. Three test runs were performed: the first was treated with the UHT-process and packed into 1 l TetraBrik carton packages with no lactase addition (= unhydrolysed), the second was prehydrolysed with 0.09% lactase in milk in a tank at 5-10°C for 20 h before UHT-treatment and packed in similar 1 l carton packages (= prehydrolysed), and the third was hydrolysed after the UHT-treatment in packages. The enzyme was added aseptically into the package (0.003%) (= posthydrolysed). The cartons were divided into four groups to be stored in the dark at temperatures of 5, 22, 30 and 45°C. The cartons were stored for 12 weeks and samples were taken for analyses at least every 4 weeks. The samples were frozen at –70°C and then transferred to –21°C for storage. The direct UHT treatment for all three types of milk was identical. In a shelf life study all the test milks were produced in the Turenki UHT-plant from the same milk batch in order to minimize variation due to raw material in unhydrolysed, prehydrolysed and posthydrolysed UHT-milks.

Description of the UHT-process (Publications IV, V, VI), Figure 6
The UHT-treatment of milks was carried out by a direct UHT process based on infusion technology (APV, Denmark). Milk was first preheated to 75±2°C for about 20 s in plate heat exchangers and then heated very rapidly to 141±2°C by steam infusion. After a holding time of 2-4 s the milk was first cooled in a flash vessel to 70±2°C, homogenised with 180/50 bar and then cooled in a plate heat exchanger to 20°C.

UHT-milks with reduced carbohydrate content (Publication VI), Figure 6
Test milks: 1. lactose unhydrolysed milk (UM) was normal skim milk received at the Valio UHT-plant, Turenki, Finland. 2. lactose hydrolysed milk (HM) was skim milk prehydrolysed in a tank. 3. carbohydrate reduced and hydrolysed milk (CRHM) was prepared using chromatographic separation to remove lactose from evaporated milk as described by Harju (1990). Separation was performed in a column at 65±2°C using water as eluate. Retention time for the protein fraction was 2.5-3.5 hours. Skim milk and water were added to balance the composition so that the milk had a normal protein content and 3.1% of lactose prior to hydrolysis. 4. carbohydrate free milk (CFM) was prepared by removing lactose almost totally by chromatographic separation. The same dosage of lactase per lactose quantity (1.9% of lactose) was used in all hydrolysed test milks. Hydrolysis time was 15 h at 5-10°C. All milks were pumped through a direct-UHT process as described above and packed into 1 l TetraBrik cartons. The direct UHT treatment for all four test milks was identical.

The cartons were divided into four groups to be stored in the dark at temperatures of 5, 22, 30 and 45°C. The cartons were stored for 12 weeks and samples were taken for analyses at least every 4 weeks. The samples were frozen at –70°C and then transferred to –21°C for storage.

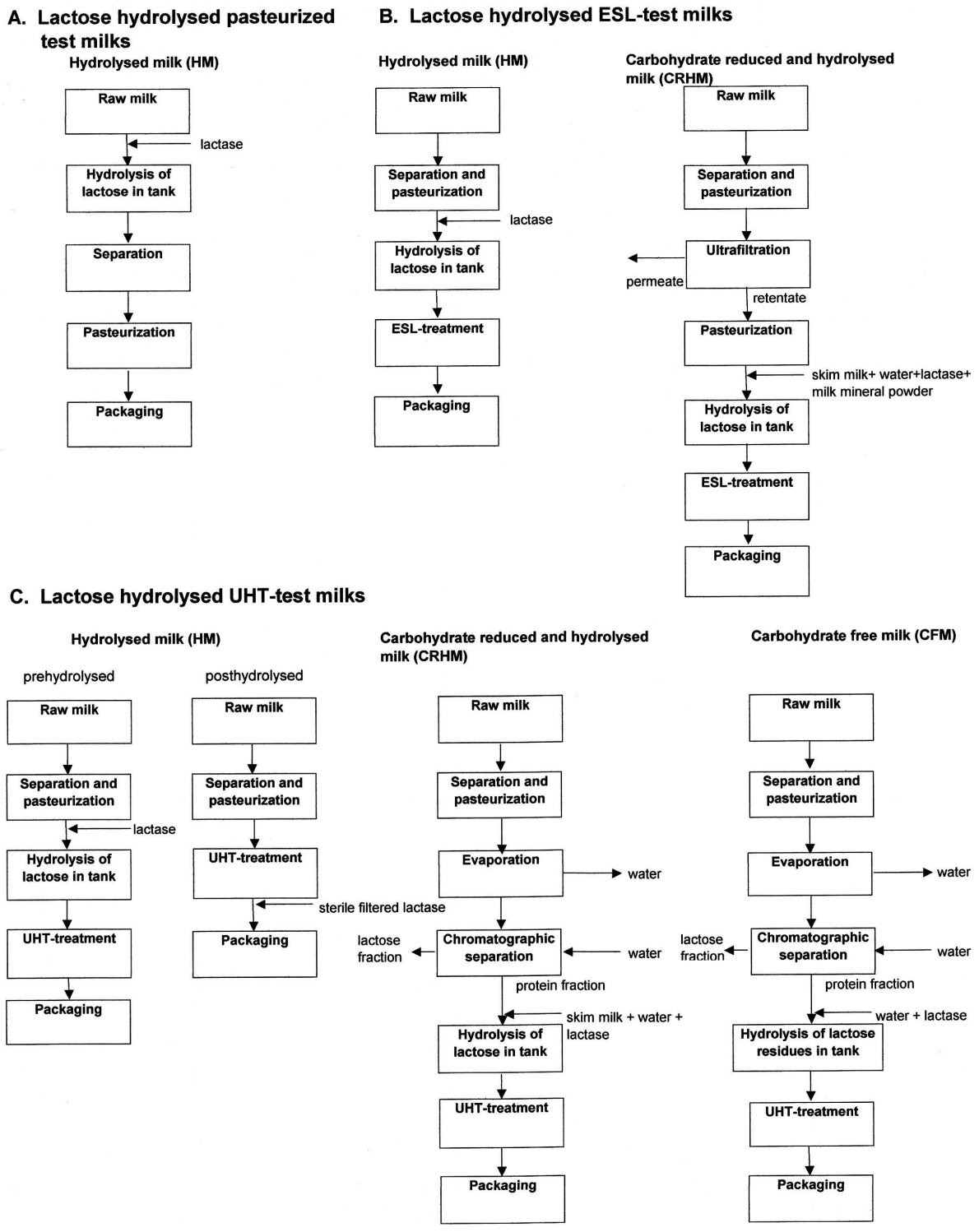


Figure 6. Manufacture of lactose hydrolysed test milks. Corresponding lactose unhydrolysed test milks were produced with the same process but without the lactase addition and hydrolysis stages.

2.3. Chemical analyses

Furosine was analysed according to the IDF standard (2004). pH was measured with a Mettler Delta 320 pH meter (Mettler-Toledo Ltd, Halstead, UK). Lactose, glucose, galactose, fructose and lactulose were measured using high-performance anion-exchange chromatography with pulsed amperometric detection with a modified method of de Slegte (2002). Lysine blockage was estimated on the basis of furosine according to Finot et al. (1981) and Evangeliste et al. (1999). Furosine values were corrected by multiplying by 0.8 before applying the Finot principle as proposed by Evangelisti et al. (1999). Reactive lysine was analysed spectrophotometrically by the o-phthaldialdehyde method according to Vigo et al. (1992) after dissolving liquid milk samples in 10% SDS for at least 1 h. Possible interference of the free amino groups of amino acids, amines and small peptides was checked in the supernatant of samples dissolved in pH 9.0 sodium tetraborate buffer solution after precipitation with 10% trichloroacetic acid solution (Naranjo et al., 1998; Goodno et al., 1981). α-Amino-N was analysed according to Lieske and Konrad (1977). Free tyrosine equivalent was analysed as described by Matsubara et al. (1958). Milk samples were analysed for fat (IDF, 1996), protein and total nitrogen (IDF, 2001), ash (IDF, 1992), total solids (IDF, 1987) and lactose (Boehringer Mannheim, 2005). In carbohydrate reduced milks the carbohydrate content was calculated as the difference between dry matter and the sum of protein, fat and ash contents.

SDS-PAGE analysis was carried out according to Laemmli (1970) by using ready made 18% Tris-HCl polyacrylamide gels (Bio-Rad, Hercules, USA). The amount of protein added to each sample well was 10 µg. Protein bands were stained with Coomassie G-250 (GelCode Blue Stain Reagent, Pierce, USA) and compared with molecular weight markers (Prestained SDS-PAGE standards, broad range, Bio-Rad, USA).

Lactase activity was determined using skim milk as substrate. The amount of glucose formed in the hydrolysis was determined and the activity of the enzyme was calculated. Lactase enzyme was diluted with 0.1 M KH_2PO_4 buffer solution at pH 7. 0.5 ml of enzyme dilution was mixed with 4.5 ml of skim milk temperated at 37°C. The mixture was incubated for 10 minutes at 37°C. The hydrolysis reaction was stopped by adding 1 ml of 4.2% (w/v) perchloric acid. After cooling to room temperature the sample was centrifuged at 3000 rpm for 10 min. After this the glucose quantity was determined from the supernatant with the glucose oxidase - peroxidase method. The 0-sample was prepared by first temperating 4.5 ml of skim milk for 10 minutes at 37°C and then adding 1 ml of 4.2% perchloric acid and finally 0.5 ml of enzyme dilution. The sample was centrifuged as described earlier and the supernatant was collected.

Roche GLU, Glucose/GOD-Perid-method kit (Boehringer Mannheim, Germany) was used for glucose determination with a slight modification of the method described on the package. To 0.1 ml of supernatant 5 ml of solution 2 (glucose oxidase $\geq$ 8 U/ml, peroxidase $\geq$ 0.35 U/ml, ABTS colour reagent 1 g/l) was added from the package and mixed. After incubation for 30-50 minutes at room temperature, absorbance was measured at a wavelength of 640 nm. Glucose quantity was calculated on basis of absorbances and the activity of the enzyme in U/g = µmol min^{-1} g^{-1} and in µkat g^{-1} (1 kat = mol s^{-1}) was calculated on basis of equation (1).

$$\frac{C_{glu} * K * L}{m_{enz} * t} = \text{activity } (\mu\text{mol min}^{-1}\ \text{g}^{-1}) \quad (1)$$

Where C_{glu} = glucose concentration of the supernatant (μmol cm^{-3})
K = correction coefficient, ratio between the final volume and the volume of reaction solution
L = dilution coefficient of the enzyme
m_{enz} = enzyme solution (g cm^{-3})
t = reaction time (min)

2.4. Microbiological analyses

Milk samples for microbiological analyses were analysed for standard plate count (ISO, 2003), coliforms (IDF, 1998), *Bacillus cereus* (ISO, 2004), psychrotrophs (ISO, 2001) and thermophilic bacteria (Frank et al., 1992).

2.5. Sensory analyses

Sensory analysis according to IDF 99C standard (1997) was used to compare UHT-milk samples stored at different temperatures. The scale ranged from 1 to 5 points. Points 3, 4 and 5 meant acceptable product quality. The product was comparable to the reference or differed from it slightly. Less than 3 points meant that the quality was not acceptable. Test milk stored at 5°C was used as a reference (=5 points). It was observed earlier that its quality changed only very slightly. A trained panel of three members was used for the sensory analysis.

2.6. Other analyses

Colour formation in milks was measured with a colorimeter (Minolta CR-210, Japan). The instrument detects the light reflected by the sample and presents the result in the CIELab colour system where the colour is described by three dimensions L (lightness), a and b (chromaticity coordinates). The colour change index (ΔE) was calculated by the equation

$$\Delta E = \sqrt{(L_t - L_0)^2 + (a_t - a_0)^2 + (b_t - b_0)^2} \quad (2)$$

where t denotes the storage time and 0 the beginning of the storage (CIE, 1974). A white standard plate was used for calibration of the device. A 1 cm layer of the sample was poured on a petri dish for the measurement and the analysis was carried out at room temperature. Measurements were performed with six repetitions.

Temperature was measured with a calibrated thermometer.

Sediment formation from UHT-packages during the shelf life study was measured as the hight of the sediment layer in mm after pouring the fluid liquid from the 1 l package.

2.7. Statistical analyses

Statistical analyses were performed with analysis of variance ANOVA and post-hoc comparisons with Duncan's test using Statistica 7.1 software (StatSoft, Inc., USA).

3. Results and discussion

3.1. Effect of lactose hydrolysis on furosine formation and available lysine in pasteurized milk (II)

Commercial products
Commercial lactose unhydrolysed, low lactose and lactose free milk samples were collected from various cities in the EU (Publication II, Table 1). The furosine contents of the milks varied from 1.6 to 198 mg/kg (4.8 – 600 mg/100 g protein). The lowest values were in pasteurized unhydrolysed milks and the highest in lactose hydrolysed UHT-milks. In ESL-milks furosine levels were close to those of pasteurized milks. The highest furosine levels found were even higher than those reported by Evangelisti et al. (1999) (furosine 555 mg/100 g protein). Thus significant protein deterioration was found in some commercial products. Highest levels of estimated blocked lysine in commercial samples in this study were at the same level as reported by Evangelisti et al. in commercial lactose free dietetic milks stored at ambient temperature. Mendoza et al. (2005) measured furosine levels of 375 and 423 mg/100 g protein in two commercial lactose hydrolysed UHT-milks. Messia et al. (2007) studied furosine from commercial lactose hydrolysed UHT-milks from Sweden, Canada, Spain, Dominican Republic and Italy and found furosine levels of 162 – 603 mg/100 g protein indicating the wide variation in heat treatments of the milks. Results of commercial milks in this study are in accordance with those of other studies.

Furosine
Pasteurization of milk has not previously been reported to cause significant blockage of lysine. In this study pasteurization of unhydrolysed raw milk caused no remarkable change in furosine in the temperature range of 60-90°C. The values were between 5.8 and 8.8 mg/100 g protein, which are in agreement with the results of Resmini et al. (1993). Resmini et al. reported furosine levels of 5-8 mg/100 g protein for pasteurized milk and 4-5 mg/100 g protein for raw milk. In this study the furosine level in raw milk was 5.4 mg/100 g protein. Resmini et al. (1990) reported 3.5-4.8 mg/100 g protein for pasteurized milk (72-90°C, 16 s) and 3.0-3.2 mg/100 g protein for raw milk. Van Renterghem and De Block (1996) measured furosine levels of 4-7 mg/100 g protein in pasteurized milk. In 10 raw farm milk samples they measured furosine levels of 4-5 mg/100 g protein and thus in pasteurized unhydrolysed milk the changes were almost non-existent.

However, in this study furosine values of 11.5-17.3 mg/100 g protein were measured in lactose hydrolysed pasteurized milk. When a trendline is applied to the Figure 1 in Publication II the following equations are obtained for furosine formation during pasteurization (15 s): $FUR = 0.187*T_p - 0.642$ for lactose hydrolysed milk and $FUR=0.0179*T_p+6.054$ for unhydrolysed milk. FUR = furosine content (mg/100 g protein) and T_p = pasteurization temperature (°C). This shows that the coefficient of the line is tenfold higher for lactose hydrolysed milk than for unhydrolysed milk. Between 75 and 90°C furosine levels were approximately doubled as compared to unhydrolysed milk. Messia (2007) measured a furosine level of 10.9 mg/100 g protein for lactose hydrolysed pasteurized milk, which is very similar to the values reported in this study. Levels of monosaccharides were similar in both studies. Overall the changes in furosine were small, but the method appeared to provide a sensitive tool to analyse changes in protein quality.

Available lysine
In lactose unhydrolysed milk the nutritional value as measured with available lysine had not deteriorated, which is in accordance with earlier studies (Resmini et al., 1993; Van Renterghem and De Block, 1996). However, in lactose hydrolysed milk the level of lysine blockage increased with the pasteurization temperature over the studied range. At its highest the estimated level of lysine blockage was only about 0.5% of total lysine. Reactive lysine

analysis gave similar results. In unhydrolysed milk the level of reactive lysine remained approximately at the same level as in raw milk, but in hydrolysed milk the levels were 3 to 7% lower. Hydrolysis of raw milk did not change the amount of reactive lysine in the milk. No trend in reactive lysine was observed in hydrolysed milk with increasing pasteurization temperature, in contrast to the observation for blocked lysine.

Proteolysis
No significant differences during the storage period were observed in α-amino-N/total-N content, and neither were there significant changes in pH. The analysis method for α-amino-N/total-N may not be sensitive enough to detect the smallest changes, but overall no significant changes occurred.

3.2. Changes in furosine and proteolysis in lactose hydrolysed ESL-milks during storage (III)

Changes in ESL-heat treated milk were studied by comparing unhydrolysed milk (UM) with prehydrolysed (HM) and carbohydrate reduced and hydrolysed milk (CRHM). Microbiological quality in milks was good before the heat treatment and packaging and after it. Changes in pH during the storage at 8°C were very small and the test milks did not differ from each other.

Furosine formation and available lysine
Furosine formation in test milks was lowest in UM, second lowest in CRHM and highest in HM. During ESL heat treatment and storage the reaction kinetics of Maillard reactions depended on the carbohydrate content in both hydrolysed test milks. The furosine content of CRHM was 76% of that of HM after the ESL treatment and the carbohydrate content was 75%. During storage the formation of furosine was about 20% slower in CRHM than in HM. The concentration of monosaccharides appeared to be a limiting factor of the Maillard reaction in hydrolysed milks. The furosine contents and the level of lysine blockage were very low in all test milks throughout the storage period, reaching a maximum of 1.7% of total lysine. Reactive lysine results support this good survival of lysine in test milks. In ESL-milk directly after the heat treatment the furosine content was almost twofold that of unhydrolysed milk.

Proteolytic changes
Differences were observed in proteolytic changes. The amount of free tyrosine equivalent clearly increased during the follow-up time in all three test milks. The free tyrosine equivalent content was highest in HM and lowest in CRHM. The production technology may offer an explanation for the difference. In the production of CRHM the milk was ultrafiltrated, during which part of the NPN-fraction was lost into the permeate. The Folin reagent, which is used to detect the tyrosine equivalent content, also reacts with tryptophan and histidine residues (Peterson, 1979). During the first two weeks of storage no proteolysis was observed but later the proteolysis proceeded linearly in all test milks. There were also slight differences between the test milks in the slopes of the fitted lines indicating the rate of proteolysis. Proteolysis was weakest in CRHM and strongest in HM.

The results from SDS-PAGE analysis support the tyrosine equivalent results. The formation of γ-casein was significant in each milk, indicating the presence of plasmin activity. The size and intensity of colour of α_s- and β-casein bands decreased less in CRHM than in UM and HM. The proteolytic activity appears to have been weaker in CRHM than in UM and HM, where proteolysis was approximately at the same level. Lower proteolysis in CRHM was probably due to the higher heat treatment due to the pasteurization after UF (Figure 6, p. 42), which may have reduced the proteolytic activity in milk. Whey proteins were hydrolysed only

very slightly, if at all, during the 56 d storage. This shows that the natural proteinase in milk, plasmin, is a significant factor for the proteolytic changes in ESL-milk.

3.3. Sensory quality during storage and shelf life of lactose hydrolysed UHT-milks

Sensorial quality of UHT-test milks was studied in order to determine the shelf life of lactose unhydrolysed or hydrolysed skim milk and which factors limit the shelf life first. Furthermore it was studied whether it is better to perform the hydrolysis before or after the UHT-treatment.

All the test milks were produced in the Turenki UHT-plant from the same milk batch in order to minimize variation due to raw material in unhydrolysed, prehydrolysed and posthydrolysed UHT-milks. Composition and quality of the raw material milks is shown in Table 5.

Table 5. Composition and quality of the raw material skim milk before the UHT-treatment.

	Unhydrolysed	Prehydrolysed	Posthydrolysed
Composition			
Protein (%)	3.6	3.5	3.6
Lactose (%)	4.4	<0.1	4.4
Glucose (%)		2.5	
Galactose (%)		2.4	
Ash (%)	0.8	0.8	0.8
Fat (%)	0.1	0.1	0.1
Microbial quality			
Std plate count (cfu/g)	48 000	22 000	48 000
Psychrotrophic (cfu/g)	88 000	31 000	88 000
Coliforms (cfu/g)	70	1 800	70
Bacillus cereus (cfu/g)	5	2	5
Thermophilic bacteria (cfu/g)	3	5	3

Results of the sensory analysis of each UHT-milk during storage at different temperatures are shown in Figures 7 (a-c). Unhydrolysed and posthydrolysed milks were very similar during the storage at different temperatures. Prehydrolysed milk had the lowest scores at all temperatures studied when compared to unhydrolysed and posthydrolysed milk. It also reached the unacceptable level of 3 points earlier at all three temperatures than unhydrolysed or posthydrolysed milks. This is probably due to the enhanced Maillard reaction and the high lactase dosage, which had to be used when hydrolysing lactose before the UHT-process.

The microbiological quality of the prehydrolysed milk before the UHT-treatment was similar to that of unhydrolysed or posthydrolysed milks except for coliforms, which were higher in prehydrolysed milk. However, all milks maintained a score above 3 points at 22°C for only 6-8 weeks. As unhydrolysed milk maintained its quality for only 8 weeks, the effect of the lactase was not very great and the plasmin system and enzymes from the contaminating microbes in milk must have been active during the storage. It is well known that gentle heat treatments in direct UHT-process do not totally inactivate enzymes in milk (Snoeren et al., 1979; Newstead et al., 2006; Datta and Deeth, 2001).

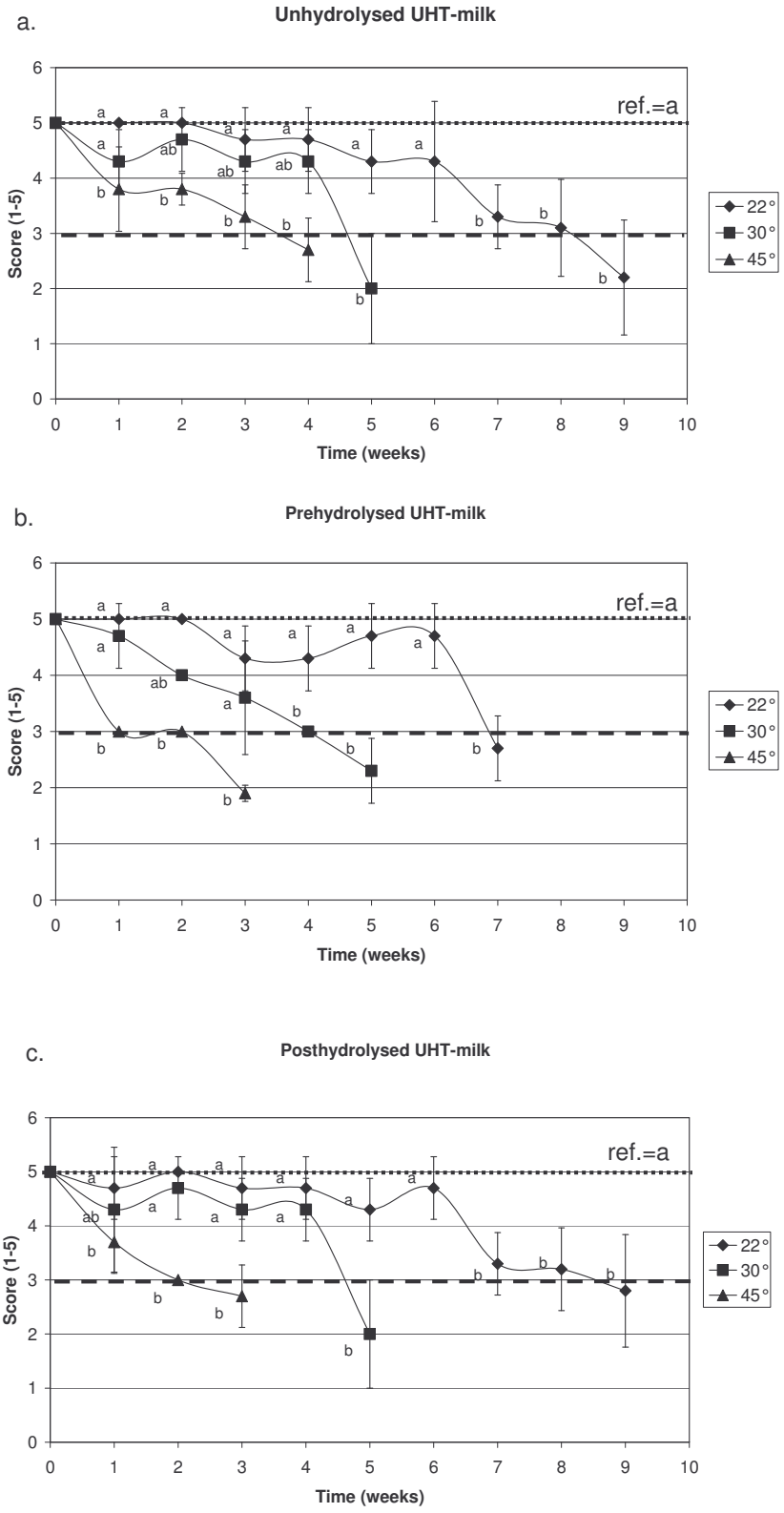


Figure 7(a-c). Sensory evaluation of the UHT-milk samples stored at different temperatures and compared to the reference (= 5 points = dotted line). Values at certain time points marked with different letters differed significantly ($p<0.05$). Dashed line = acceptance limit.

Comments for the quality defects observed are given in Table 6. The first quality defects noticed in unhydrolysed and posthydrolysed milk at 22°C in the sensory evaluation were off-flavours such as cooked, heated, burned flavour, later untypical taste and bitterness. At 30 and

45°C colour changes were noticed as well as off-flavours. Colour changes noticed were first yellowness, light brown or reddish and later more intense brownish shades.

In prehydrolysed milk at 22°C the first quality defects noticed were colour change, cooked flavour and slight bitterness, indicating that the Maillard reaction had already proceeded, unlike in unhydrolysed and posthydrolysed milk. At 30°C the changes came early, with descriptions of cooked flavour, colour change and bitterness. At 45°C these changes took place very soon, making the milk visibly coloured already in one week.

Table 6. Quality defects observed in UHT-milks in the sensory panel test. Symbols: + = slight, ++ = moderate, +++ = significant, discont. = discontinued.

Unhydrolysed milk

Time (weeks)	0	1	2	3	4	5	6	7	8	9	10
22°C											
Off-flavour							+	+	++	++	discont.
Colour											discont.
Texture											discont.
30°C											
Off-flavour		+	+			+++	discont.				
Colour							discont.				
Texture							discont.				
45°C											
Off-flavour		+	+	++	++	discont.					
Colour			+	+	++	discont.					
Texture						discont.					

Prehydrolysed milk

Time (weeks)	0	1	2	3	4	5	6	7	8
22°C									
Off-flavour								++	discont.
Colour						+		++	discont.
Texture									discont.
30°C									
Off-flavour			+	+	++	+++	discont.		
Colour			+	+	++	++	discont.		
Texture							discont.		
45°C									
Off-flavour		+	++	+++	discont.				
Colour		+	++	+++	discont.				
Texture					discont.				

Posthydrolysed milk

Time (weeks)	0	1	2	3	4	5	6	7	8	9	10
22°C											
Off-flavour		+						+	+	++	discont.
Colour											discont.
Texture											discont.
30°C											
Off-flavour		+	+		+	+++	discont.				
Colour					+	++	discont.				
Texture							discont.				
45°C											
Off-flavour		++	++	+++	discont.						
Colour		++	++	+++	discont.						
Texture					discont.						

The amount of sediment measured from the packages is shown in Figure 8 (a-c). Sediment formation was similar in all test milks. The height of the sediment usually increased with storage time and was greatest in milks stored at 30°C. This was in agreement with results of Kocak and Zadow (1985b), who found that the order of resistance of UHT milk to the onset of age gelation at various storage temperatures was: 50°C, 40°C > 2°C > 10°C > 15°C > 20°C > 25°C > 30°C. The lower amount of sediment at the higher temperatures may be due to the

high degree of protein decomposition producing extensively degraded proteins which were unable to form a stable gel matrix (Manji et al., 1986). Lactose hydrolysed milks did not differ from unhydrolysed milk in the amount of sediment. At 22°C, sediment formation started after 8-9 weeks, at 30°C after 5 weeks and at 45°C after 5-8 weeks.

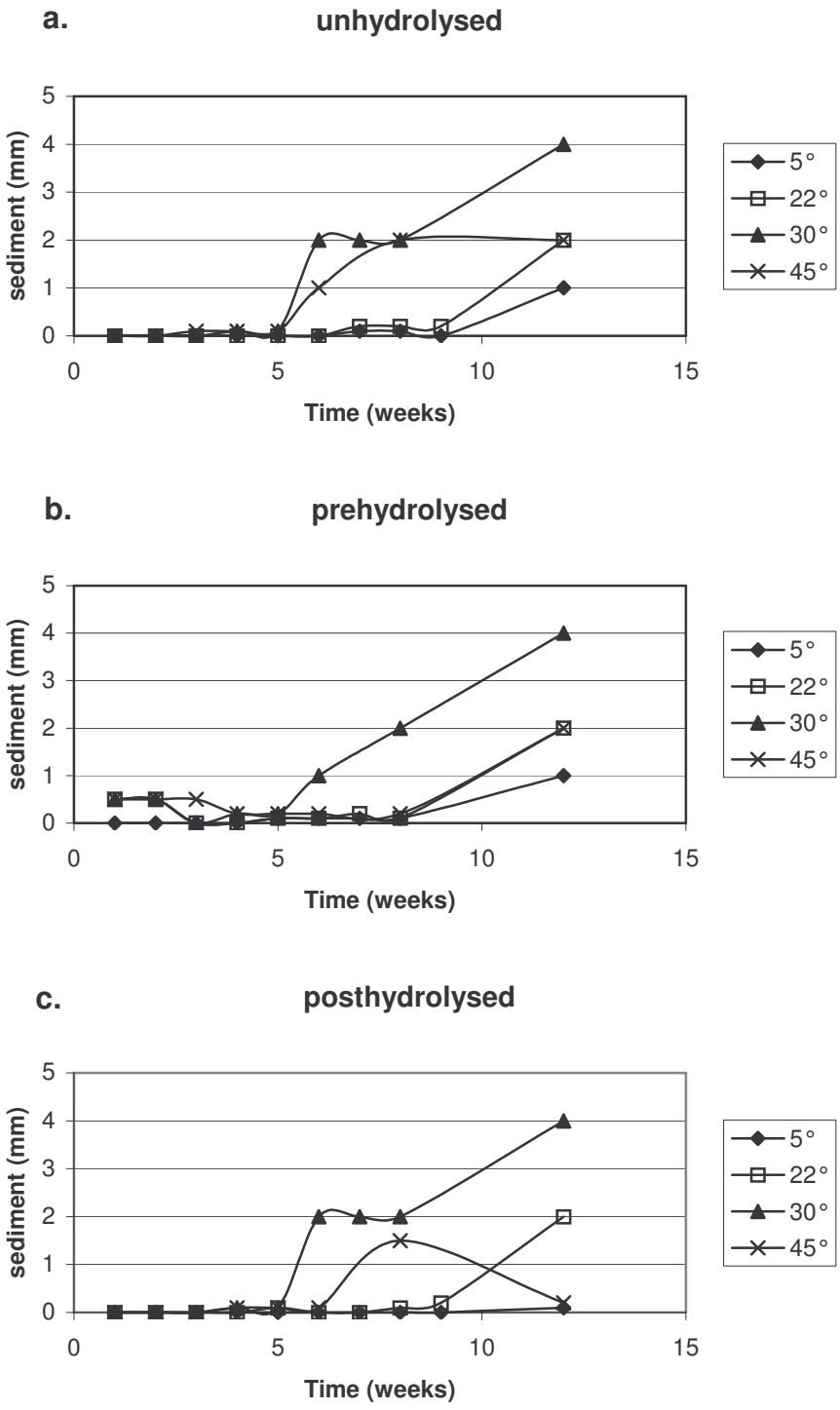


Figure 8 (a-c). Height of the sediment measured from the bottom of the package during storage.

The sensory evaluations showed that lactose hydrolysed milks were very prone to quality deterioration. Using higher heating temperatures during the UHT-process could reduce proteolytic changes, but at the same time the Maillard reaction would accelerate. Renner et al. (1986) observed statistically significant changes in sensory quality of posthydrolysed UHT-

milk milk containing 3.5% fat after storage of 16 weeks at 22°C and after 10 weeks at 38°C. UHT-process parameters were not presented. Fat in milk may have masked quality defects in the milk. Skim milk is probably clearly more prone to taste defects than milk containing 3.5% fat.

This comparison showed that prehydrolysed milk lost its sensory quality rather faster than unhydrolysed or posthydrolysed milk. The differences between unhydrolysed and posthydrolysed milks were insignificant. This indicated that plasmin enzyme and contaminating microbial enzymes also played a significant role causing changes in UM. If a degree of hydrolysis as high as in this study (about 98%) is needed, hydrolysis before the UHT-treatment requires a high dosage of enzyme. This brings a significant proteolytic contamination into the product. Although the UHT-treatment can destroy most of the proteases, proteolysis can take place already during the hydrolysis phase and continue at a reduced level after UHT-treatment. The Maillard reaction started more strongly during the UHT-process in prehydrolysed milk than in other milks. Therefore performing the hydrolysis before the UHT-treatment does not help to avoid quality problems.

3.4. Proteolytic changes in lactose hydrolysed UHT-milk during storage (IV)

UHT-test milks were monitored at four temperatures for 12 weeks in order to follow proteolytic changes during storage. Test milks were produced from the same milk batch or from milk of very similar quality. According to α-amino-N/total-N no significant proteolysis occurred at 5 or 22°C, but at 30 and 45°C it was very noticable. In lactose hydrolysed milks proteolysis was clearly more extensive than in unhydrolysed milk. There did not appear to be any difference according to whether lactose hydrolysis had been performed before or after the UHT-treatment. This can be explained by assuming that in test milk where hydrolysis was performed before the UHT-treatment the high enzyme dosage (0.09%) was largely inactivated in the heat treatment and residual proteolytic activity was approximately the same as in posthydrolysed test milk, where the dosage was small (0.003%) but all the proteolytic side activity survived.

Modler et al. (1993) described production of fluid milk with a high degree ($\geq$90%) of hydrolysis. The authors stated that hydrolysis can be performed either before the UHT-treatment or after it. The latter required dilution and sterile filtration of the enzyme before aseptic addition to the package. They also claimed that the prehydrolysis process alternative has the advantage of destroying the lactase and its possible proteolytic side activities before packing. However, this study shows that the inactivation of proteolytic side activities is not complete if a direct UHT-process is used.

Unhydrolysed and posthydrolysed milks were from the same milk batch, but prehydrolysed milk was from a different batch of very similar quality milk. The results showed that in both prehydrolysed and posthydrolysed milks the proteolysis had proceeded in a very similar way. These results were confirmed with another test in which all these three milks were prepared from the same milk batch. Proteolysis in pre- and posthydrolysed milks was very similar and higher than in unhydrolysed milk. This means that a large part but not all of the proteolytic side activities were destroyed in the UHT-process in prehydrolysed milk. In prehydrolysed milk the dosage was 0.09% and in posthydrolysed milk 0.003%. This means that approximately 97% of the proteolytic side activities of Godo YNL-2 lactase were destroyed in the UHT-process as calculated according to equation (3).

$$100*(0.09-0.003)/0.09=96.7\% \quad (3)$$

SDS-PAGE analyses showed that at 5°C no significant changes in protein bands were evident, but already at 22°C after 4 weeks some release of γ-casein was observed referring to the action of plasmin, and particularly β-casein bands were weakened in lactose hydrolysed milks. At 30 and 45°C proteolysis was already significant in all milks but especially strong in lactose hydrolysed milks.

After 12 weeks of storage proteolysis was very mild at 5°C, but very clear at all other tested temperatures. The action of plasmin was seen from the release of γ-casein in all milks, but in lactose hydrolysed milks there was additional proteolysis indicating the presence of contaminating proteolytic enzymes in the commercial lactase. After 12 weeks of storage at 45°C the caseins had totally disappeared and at 30°C only traces of casein were found. K-casein survived best of the caseins in these milks. Whey proteins were much less affected than the caseins. SDS-PAGE analyses support the α-amino-N/tot.N-results that proteolysis in lactose hydrolysed milks was more extensive than in unhydrolysed milk. In both lactose hydrolysed milks the proteolysis was of approximately same magnitude. Based on SDS-PAGE analyses the proteolysis of prehydrolysed milk may be slightly more extensive than that of posthydrolysed milk.

In lactose hydrolysed milks stored at 45°C some very large molecular compounds were observed in SDS-PAGE gels both after 4 and 12 weeks of storage. This may indicate the formation of high molecular weight Maillard products, such as melanoidins (Brands et al., 2002; O'Brien and Morrissey, 1989a; Nursten, 1981). The Maillard browning reaction has a relatively high temperature coefficient: Q_{10} for pigment formation is in the range 2 to 6 at 40-50°C (Labuza and Saltmarch, 1981).

pH of the milks was monitored during the storage period and it was found that a high correlation existed between the changes in pH and the change in α-amino-N/tot.N (r^2=0.865, n= 36). This may mean that the pH change can be used as an indicator for proteolysis. Naturally, it must be ensured that the pH-drop is not due to microbiological contamination. Mittal et al. (1988) argued that pH decrease during storage of recombined lactose hydrolysed milk was associated with the interaction between lactose and proteins, with the hydrolytic dephosphorylation of casein and with changes in the calcium-phosphorus equilibrium. The pH decrease during storage of 12 weeks at 30°C in recombined lactose hydrolysed UHT-milk containing 3% fat was 0.17 pH-units, whereas in the present study the corresponding pH-drop for lactose hydrolysed UHT-skim milk was 0.29 pH-units. The difference may be due to the higher activity of proteinases in milk of this study than in recombined milk made from medium heat milk powder in the study of Mittal et al.

Andrews et al. (1977) ascribed the decrease in pH to a loss of free ε-NH_2 group of lysine during Maillard reactions. The authors explained that the greater pH decrease in lactose hydrolysed recombined milk vs. unhydrolysed milk was due to the more intensive Maillard reactions in hydrolysed milk. In this study correlation between pH-drop and change in α-amino-N/total-N was higher (r^2=0.865, n=36) than between pH-drop and furosine change (r^2=0.809, n=36). Already Samel et al. (1971) showed that a significant breakdown of proteins takes place during storage of 13 months at 20-37°C in indirectly UHT-treated milk.

According to O'Brien and Morrissey (1989b), the pH of the Maillard reaction system decreases due to the disappearance of basic amino groups. During storage at 40°C, Corzo et al. (1994) measured a simultaneous drop in pH with significant proteolysis in indirectly UHT-treated milk made from milk contaminated with thermostable proteinases. Adler-Nissen (1986) showed that H^+-ions are released during enzymatic hydrolysis of proteins. The release is dependent on the hydrolysis temperature and pH. There are probably several parallel reactions proceeding simultaneously at elevated storage temperatures.

3.5. Furosine formation and available lysine in lactose hydrolysed UHT-milk (V)

In unhydrolysed and posthydrolysed milk the furosine contents immediately after packing were practically identical. In prehydrolysed milk the furosine content was approximately three times higher. In pre- and posthydrolysed milks the furosine content increased during the storage at the same rate, although in posthydrolysed milk the initial value was lower than in prehydrolysed milk. At 5°C and 22°C this difference was maintained for 12 weeks of storage. If milk was stored at higher than room temperature, it was practically irrelevant with regard to the level of furosine whether the hydrolysis was performed before or after the UHT-treatment. Furosine values in unhydrolysed milks remained clearly lower than in hydrolysed milks at all storage temperatures tested. Directly after UHT-treatment the furosine content in prehydrolysed milk was about threefold to that in unhydrolysed milk. Similar result was found in publication VI.

The level of lysine blockage estimated on the basis of furosine was very low at 5°C, reasonably low at 22°C, but at 30°C and higher increased rapidly to very high levels. At 22°C lysine blockage remained below 10% of total lysine in lactose hydrolysed milks for the 12 week period, but it increased continuously and was acceptable only if the storage period was limited to a maximum of 12 weeks. As was observed in the shelf life study, off-taste defects are detected already clearly before 12 weeks of storage.

The colour index change was also very small at 5°C, but some changes in lactose hydrolysed milks were detected at 22°C, as was also noticed in the sensory evaluation test. Colour index changes in milks followed the Maillard reaction, although the early stages of the Maillard reaction do not cause colour changes (Hurrell and Finot, 1983; Evangelisti et al., 1999). At higher storage temperatures the colour developed faster. A high correlation (0.926, n=36) between furosine change and colour index change during storage was found, suggesting that colour measurement could be used as an approximate estimate of furosine in lactose hydrolysed milks. The correlation was highest in milks stored at temperatures higher than 5°C. Although the early Maillard reaction in milk does not cause colour changes, it appeared that in milks in which the Maillard reaction had proceeded to advanced stages causing colour changes, the furosine content was also higher than in milks without or with significantly less Maillard reaction. The advanced Maillard reaction at 45°C was also noticed in the content of monosaccharides, which decreased during the storage most probably due to reactions with proteins and peptides. Leclère and Birlouez-Aragon (2001) reported that formation of advanced Maillard products takes place already from the beginning of the Maillard reaction and therefore estimation of lysine damage by furosine may underestimate the unavailability of lysine. Results of Henle et al. (1991) support this opinion.

Visible browning is often used as a criterion of overheating in milk-drying processes. Browning is not a sensitive but a clear indicator of protein damage in lactose hydrolysed milk processing. In a study by Leclère and Birlouez-Aragon (2001), formation of furosine and advanced Maillard products measured as FAST (fluorescence) index correlated well, as in the case of furosine and colour change in this study. However, when the Maillard reaction advanced to further stages the correlation between furosine and FAST index decreased in the study of Leclère and Birlouez-Aragon. In one study by Dehn-Müller et al. (1991), furosine and HMF values measured from 190 UHT-milk samples from 45 German dairies correlated moderately well (r=0.846). Furosine is released from early Maillard reaction products, whereas HMF rather indicates advanced reaction products.

3.6. Furosine formation and proteolytic changes in lactose hydrolysed, carbohydrate reduced milks (VI)

This work was carried out in order to study the effect of reduction of carbohydrate content in milk before the enzymatic hydrolysis of lactose on the nutritional quality of the milk. Carbohydrate reduction was made to two different levels: about 23% reduction and about 96% reduction.

Protein contents in the test milks were very similar, only in lactose hydrolysed milk (HM) was the protein slightly lower (3.19%) due to dilution during the heat treatment process. In unhydrolysed milk (UM) and HM the proteolysis proceeded as reported already in publication (V). In HM it was more extensive than in UM, but it was also clear in UM. Lactose in HM was hydrolysed before the heat treatment and therefore many of the side activities in commercial lactase were destroyed, but again despite this significantly higher proteolysis was found than in UM. In CRHM, proteolysis was approximately as extensive as in HM. This may be partly due to the normal skim milk which is part of the recipe (Figure 6, p. 42) and which brings active plasmin into the product as well as side activities of the lactase, and probably also due to the lower microbiological quality of the CRHM before the UHT-treatment.

Of the test milks proteolysis was lowest in the carbohydrate free milk (CFM) when stored at temperatures of 5-30°C. At 45°C it was significant, probably due to the thermostable contaminating proteases originating from the chromatographic separation. These proteases probably did not function at lower storage temperatures. In the chromatographic separation milk was eluted at 65°C with water. Elution time for protein fraction was 2.5-3.5 hours, after which the protein and minerals fraction was collected and cooled quickly to below 10°C. This treatment probably inactivated part or all of the plasmin enzyme system in milk, and according to SDS-PAGE and tyrosine equivalent analyses CFM stored for 12 weeks at 22°C still contained protein which was only very slightly hydrolysed and comparable to that of raw milk. At 30°C proteins were also very resistant to proteolysis. In HM and CRHM the proteins were very clearly hydrolysed even after 4 weeks of storage at 22 and 30°C.

Reduction of lactose in CRHM by 23% before its hydrolysis reduced furosine formation by 9-24% compared to HM, depending on the storage temperature, showing that furosine formation was related to the quantity of reducing sugars. The fact that the reduction was not greater was probably due to the heat treatment during chromatographic separation. Furosine in CRHM was slightly lower than in HM but significantly higher than in UM or in CFM. In CFM the furosine formation was avoided during storage almost completely. The furosine remained at its original level and did not increase during the storage at any temperature tested. This meant that the estimated blocked lysine level was lowest in CFM. Again, correlation between colour index change and furosine change during the storage was high (0.913, n=60), although the compositions of the milks varied. However, it should be noted that the correlation was better the higher was the storage temperature. At 5°C the correlation was very poor due to negligible changes in furosine and colour.

CFM with artificial sweetener (asesulfame K) was manufactured and stored in the same way as CFM. Furosine formation and proteolysis during storage were very similar to that of CFM.

By removing lactose chromatographically from skim milk at 65°C it was possible to inactivate the plasmin enzyme system in milk and at the same time to avoid the Maillard reaction between reducing sugars and free amino groups. The low dosage of lactase needed to hydrolyse lactose residues brought less contaminating proteolytic enzymes to the product. This can provide a new method to produce low lactose or lactose free milk drinks with a long shelf life, minimal Maillard reactions and good nutritional protein quality. A patent has been

applied for this method. However, further research is needed to optimize the process in terms of sensory quality, composition of product and production costs.

3.7. Comparison of different processes

Figure 9 shows the formation of furosine in different heating processes tested in this study. The results show logically that furosine content increased as the heat treatment intensified. Reduction of the molar content of reducing sugars helped to avoid formation of furosine.

During storage, proteolysis was best avoided in pasteurized and ESL-milks, mostly due to the low storage temperatures (5 and 8°C). In UHT-milks proteolysis was best avoided in CFM, in which the Maillard reaction was also best avoided.

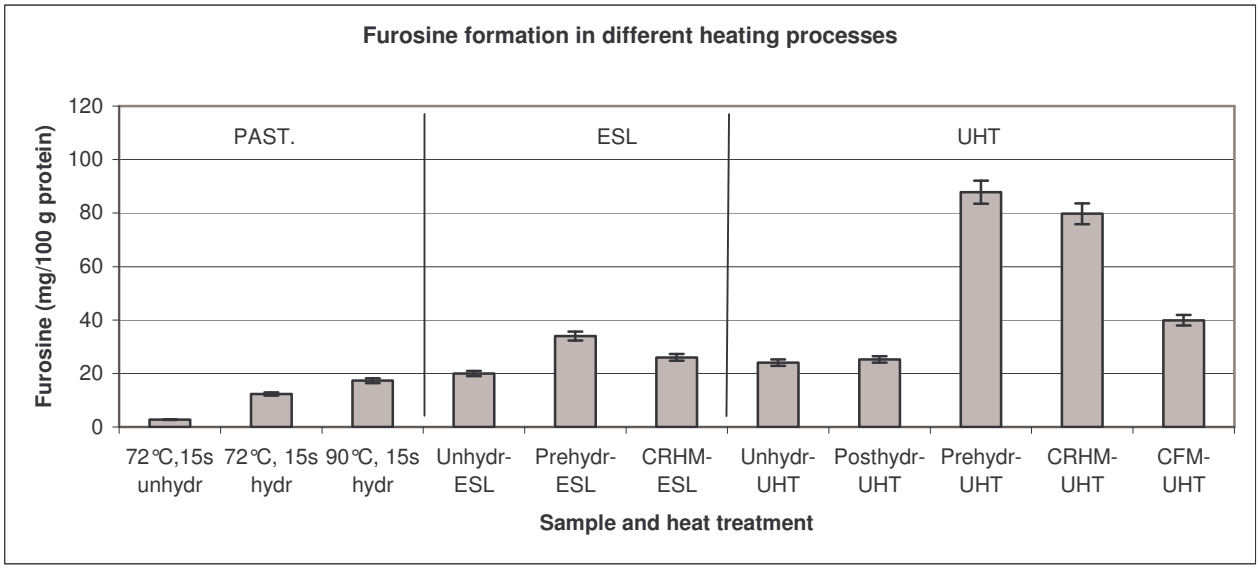


Figure 9. Summary of furosine in milks heat treated in different ways. Furosine was analysed immediately after the heating process. Symbols: unhydr = lactose unhydrolysed, hydr=lactose hydrolysed, ESL=ESL heat treatment, Prehydr= lactose prehydrolysed, posthydr=lactose hydrolysed aseptically in the package, CRHM= carbohydrate reduced lactose hydrolysed milk, CFM=carbohydrate free milk.

4. Conclusions

Vulnerability of lactose hydrolysed milk to Maillard reactions during heating is a well-known problem. Maillard reactions reduce the nutritional quality of milk proteins and easily cause sensory quality defects in the product. In this study the effect of Maillard reactions on the nutritional value of milk was observed already in mild pasteurization treatments at 60-90°C. Maillard reactions limit especially the shelf life of lactose hydrolysed UHT-milks. In lactose free milks, which have a very low residual lactose limit in several countries, this problem is even more acute.

In UHT-milks there is also a problem with proteolysis caused by indigenous proteinases or microbial proteinases from contaminating microbes. In lactose hydrolysed milk there is usually also contamination from lactase preparations, which generally contain proteolytic and other side activities. In normal, unhydrolysed UHT-milk the proteolytic side activities can be destroyed by intensified heat treatment during the UHT-process. In lactose hydrolysed milk this is not usually possible due to enhanced Maillard reactions, or if lactase is added after the UHT-treatment the proteolytic side activities of the enzyme may cause quality defects during storage at ambient temperature. Performing the hydrolysis of lactose before or after the UHT-treatment was compared using milks with a high degree of hydrolysis (~98%). Both alternatives caused quality problems in UHT-milk during storage at room or higher temperature, but defects were somewhat more evident in prehydrolysed milk.

In carbohydrate reduced hydrolysed milks the severity of Maillard reactions decreased in proportion to the decreasing molar concentration of the residual reducing sugars, glucose and galactose. Together with the normal taste of milk this is a significant improvement for lactose malabsorbing consumers, who generally stop drinking milk when the symptoms of intolerance appear. This also means less browning during cooking with carbohydrate reduced hydrolysed milk compared to traditional hydrolysed milk. Carbohydrate reduced hydrolysed milk had a lower furosine level than traditional lactose hydrolysed milk and particularly in ESL-milk the proteolytic changes were also minimal.

The available lysine estimated on the basis of furosine gave very repeatable results. The estimated available lysine during storage decreased most in lactose hydrolysed UHT-milks, especially when stored at 30°C or 45°C. In lactose hydrolysed pasteurized or ESL-treated milks the reduction in available lysine was insignificant. However, on the basis of information from the literature the lysine blockage may have been underestimated, because the furosine method does not take into account the advanced phases of the Maillard reaction. Thus the results shown may represent the minimum loss of lysine in milk.

Chromatographic separation in the production of lactose reduced or lactose free milk provides new possibilities to avoid Maillard reactions and to inactivate the plasmin enzyme system in milk at the same time. Carbohydrate free milk, from which carbohydrates were almost totally removed, had a better storage stability than milks which were manufactured in the traditional way. Proteolytic changes during storage were significantly smaller than in other milks. However, further studies are needed to optimize the process in terms of sensory quality, composition of product and production costs.

About 70% of the world population are lactose malabsorbers, who normally stop using milk when the symptoms of lactose intolerance appear. The new technologies make it possible to produce milk products with normal taste and high nutritional protein quality for lactose intolerant people around the world. These people can again benefit from the high nutritional value of milk.

5. References

Adler-Nissen, J., Enzymic Hydrolysis of Food Proteins. Elsevier Applied Science Publishers, London 1986, p. 11, 123

Andersson, I., Öste, R., Nutritional quality of heat processed liquid milk. In: Heat- induced changes in milk (ed. P. F. Fox), IDF, Brussels 1995, pp. 279-301.

Andrews, A. T., Brooker, B. E., Hobbs, D. G., Properties of aseptically packed ultra-heat-treated milk. Electron microsopic examination of changes occurring during storage, J. Dairy Res. 44 (1977) 283-292.

Anon., Lysinoalanin in hitzebehandelten Milcherzeugnissen, Bundesgesundheitsblatt 29 (5) (1986) 166-168.

Anon., National Enzyme Company, technical information: Dairyzime sTM, http://www.nzimes.com/dairyzimes/doc_folder/Dairyzimes_tecsheet.pdf, 20.9.2007.

Ashoor, S. H., Zent, J. B., Maillard reaction of common amino acids and sugars, J. Food Sci. 49 (1984) 1206-1207.

Asp, N. G., Low intestinal lactase activity – a 40 years perspective, Scand. J. Nutr. 45 (4) (2001) 154-155.

Ateş, S., Mehmetoğlu, Ü., A new method for immobilization of β-galactosidase and its utilization in a plug flow reactor, Process Biochem. 32 (5) (1997) 433-436.

Bakken, A. P., Hill, Jr., C. G., Amundson, C. H., Hydrolysis of lactose in skim milk by immobilized β-galactosidase (*Bacillus circulans*), Biotechn. Bioeng. 39 (1992) 408-417.

Berg, H. E., van Boekel, M A. J. S., Degradation of lactose during heating of milk. 1. Reaction pathways, Neth. Milk Dairy J. 48 (1994) 157-175.

Birlouez-Aragon, I., Effect of lactose hydrolysis on calcium absorption during duodenal milk perfusion, 28 (6A) (1988) 1465-1472.

Birlouez-Aragon, I., Effects of lactose hydrolysis on galactose metabolism: influence on lens transparency, IDF Bulletin 289 (1993) 65-71.

Birlouez-Aragon, I., Nicolas, M., Metais, A., Marchond, N., Grenier, J., Calvo, D., A rapid fluorimetric method to estimate the heat treatment of liquid milk, Int. Dairy J. 8 (1998) 771-777.

Birlouez-Aragon, I., Stevenin, L., Rouzier, C., Brivet, M., Lactose intake and lactase activity: two risk factors for senile and diabetic cataract, Age and Nutrition 1 (2) (1990) 74-79.

Blanck, B., Flückiger, E., Rüegg, M., Steiger, G., Veränderungen biochemischer, physkalischer, technologisher und sensoricher Merkmale von UHT-Milch im Verlaufe der Lagerung, alimenta-Sonderausgabe (1980) 27-47.

Bleumink, E., Young, E., Identification of the atopic allergen in cow's milk. Int. Arch. Allergy 34 (1968) 521-543.

Boehringer Mannheim, Lactose/D-glucose UV-method for the determination of lactose and D-glucose in foodstuffs and other materials, (2005) Cat. No. 10 986 119 035, 4 p.

Brands, C.M.J., Alink, G. M., van Boekel, M. A. J. S., Jongen, W. M. F., Mutagenicity of heated sugar-casein systems: Effect of the Maillard reaction, J. Agric. Food Chem. 48 (2000) 2271-2275.

Brands, C. M. J., van Boekel, M. A. J. S., Reactions of monosaccharides during heating of sugar-casein systems: building of a reaction network model, J. Agric. Food Chem. 49 (2001) 4667-4675.

Brands, C. M. J., van Boekel, M. A. J. S., Kinetic modeling of reactions in heated monosaccharide–casein systems, J. Agric. Food Chem. 50 (2002) 6725-6739.

Brands, C. M. J., Wedzicha, B. L., van Boekel, M. A. J. S., Quantification of melanoidin concentration in sugar-casein systems, J. Agric. Food Chem. 50 (2002) 1178-1183.

Broome, M. C., Roginski, H., Hickey, M. W., The enzymic hydrolysis of lactose in skim milk yoghurt, Aust. J. Dairy Technol. 38 (1983) 35-37.

Burbrink, C. N., Hayes, K. D., Effect of thermal treatment on the activation of bovine plasminogen, Int. Dairy J. 16 (2006) 580-585.

Burvall, A., Asp, N.-G., Bosson, A., San José, C., Dahlqvist, A., Storage of lactose-hydrolysed dried milk: effect of water activity on the protein nutritional value, J. Dairy Res. 45 (1978) 381-389.

Burvall, A., Asp, N.-G., Dahlqvist, A., Öste, R., Nutritional value of lactose-hydrolysed milk: protein quality after some industrial processes, J. Dairy Res. 44 (1977) 549-553.

Bury, D., Jelen, P., Lactose hydrolysis using a disrupted dairy culture: Evaluation of technical and economical feasibility, Can. Agric. Eng. 42 (2) (2000) 75-80.

Calvo, M. M., de la Hoz, L., Flavour of heated milks. A review, Int. Dairy J. 2 (1992) 69-81.

Carpenter, K. J., The estimation of available lysine in animal-protein foods, Biochem. J. 77 (1960) 604-610.

Carrara, C. R., Rubiolo, A. C., Determination of kinetic parameters for free and immobilized β-galactosidase, Process Biochem. 31 (3) (1996) 243-248.

Cerlesi, P., Method and plant for hydrolysis of lactose in milk with an enzyme fixed on a support. EP Appl. 0997071 (2000).

Chen, K-C., Houng, J-Y., Ling, A. C., Product inhibition of the enzymatic hydrolysis of lactose, Enzyme Microb. Technol. 7 (1985) 510-514.

Chen, L., Daniel, R. M., Coolbear, T., Detection and impact of protease and lipase activities in milk and milk powders, Int. Dairy J. 13 (2003) 255-275.

Choi, S. H., Lee, S.-B., Won, H.-R., Development of lactose hydrolysed milk with low sweetness using nanofiltration, Asian-Aust. J. Anim. Sci. 20 (6) (2007) 989-993.

CIE Colorimetry Committee, J. Opt. Am. 64 (1974) 896-897.

Claeys, W. L., Van Loey, A. M., Hendrickx, M. E., Kinetics of hydroxymethylfurfural, lactulose and furosine formation in milk with different fat content, J. Dairy Res. 70 (2003) 85-90.

Collins, S. J., Bester, B. H., McGill, A. E. J., Influence of psychrotrophic bacterial growth in raw milk on the sensory acceptance of UHT skim milk, J. Food Prot. 56 (5) (1993) 418-425.

Corzo, N., López-Fandiño, R., Delgano, T., Ramos, M., Olano, A., Changes in furosine and proteins of UHT-treated milks stored at high ambient temperatures, Z. Lebensm. Unters. Forsch. 198 (1994) 302-306.

Cousin, M. A., Presence and activity of psychrotrophic microorganisms in milk and dairy products: A review, J. Food Protect. 45 (2) (1982) 172-207.

Dahlqvist, A., Digestion of lactose. In: Milk Intolerances and Rejection (Ed. J. Delmont), Karger, Basel 1983, pp. 11-16.

Dahlqvist, A., Asp, N.-G., Burvall, A., Rausing, H., Hydrolysis of lactose in milk and whey with minute amounts of lactase, J. Dairy Res. 44 (1977) 541-548.

Dahlqvist, A., Mattiasson, B., Mosbach, K., Hydrolysis of β-galactosidases using polymer-entrapped lactase. A study towards producing lactose free milk, Biotechn. Bioeng. 15 (1973) 395-402.

Dariani, D. N., Frank, J. F., Loewenstein, M., Manufacture of low lactose yoghurt by simultaneous lactose hydrolysis and bacterial fermentation, Cultured Dairy Prod. J., 17 (1982) 18-19, 22.

Datta, N, Deeth, H. C., Age gelation of UHT milk – A review, Trans IChemE 79 Part C (2001) 197-210.

Datta, N., Deeth, H. C., Diagnosing the cause of proteolysis in UHT milk, LWT 36 (2003) 173-182.

Datta, N., Elliott, A. J., Perkins, M. L., Deeth, H. C., Ultra-high-temperature (UHT) treatment of milk: comparison of direct and indirect modes of heating, Aust. J. Dairy Technol. 57 (3) (2002) 211-227.

Davis, C. D., Snyderwine, E. G., Protective effect of N-acetylcysteine against heterocyclic amine-induced cardiotoxicity in cultured myocytes and in rats, Food Chem. Toxicol. 33 (8) (1995) 641-651.

Dehn-Müller, B., Müller, B., Erbersdobler, H. F., Untersuchungen zur Proteinschädigung in UHT-Milch, Milchwissenschaft 46 (7) (1991) 431-434.

Dehn-Müller, B., Müller, B., Lohmann, M., Erbersdobler, H. F., Determination of furosine, lysinoalanine (LAL) and 5-hydroxymethylfurfural (HMF) as a measure of heat intensity for UHT-milk. In: Milk proteins: nutritional, clinical, functional and technological aspects (eds. C.A. Barth, E. Schlimme), Dr. Dietrich Steinkopff Verlag, Darmstadt 1989, pp. 228-232.

De Rafael, D., Villamiel, M., Olano, A., Formation of lactulose and furosine during heat treatment of milk at temperatures of 100-120°C, Milchwissenschaft 52 (2) (1997) 76-78.

De Slegte, J. Determination of trans-galacto-oligosaccharides in selected food products by ion-exchange chromatography: collaborative study, J. AOAC Int. 85 (2) (2002) 417-423.

Desrosiers, T., Savoie, L., Bergeron, G., Parent, G., Estimation of lysine damage in heated whey proteins by furosine determinations in conjunction with the cell digestion technique, J. Agric. Food Chem. 37 (1989) 1385-1391.

De Vrese, M., Physiological and metabolic effects of consuming hydrolysed lactose products, IDF Bulletin 289 (1993) 62-64.

Dinelli, D., Marconi, W., Morisi, F., Fibre-entrapped enzymes. Methods Enzymol. 44 (1976) 227-243.

Driessen, F. M., Inactivation of lipases & proteinases (indigenous & bacterial), IDF Bulletin 238 (1989) 71-93.

DSM WO 2007/060247 Enzyme preparations yielding a clean taste (2007).

Dworschák, E., Hegedüs, M., Effect of heat treatment on the nutritive value of proteins in milk powder, Acta Alim. 3 (3) (1974) 337-347.

Eichner, K. The influence of water content on non-enzymic browning reactions in dehydrated foods and model systems and the inhibition of fat oxidation by browning intermediates. In: Water relations of foods. International symposium 1974 (Ed. R. B. Duckworth), Academic Press, London 1975, p. 417-434.

Elliott, A. J., Datta, N., Amenu, B., Deeth, H. C., Heat-induced and other chemical changes in commercial UHT milks, J. Dairy Res. 72 (2005) 442-446.

Elliott, A. J., Dhakal, A., Datta, N., Deeth, H. C., Heat-induced changes in UHT milks – Part 1, Aust. J. Dairy Technol. 58 (1) (2003) 3-10.

Enattah, N. S., Sahi, T., Savilahti, E., Terwilliger, J. D., Peltonen, L., Järvelä, I., Identification of variant associated with adult-type hypolactasia, Nature Genetics 30 (2) (2002) 233-237.

Enright, E., Bland, A. P., Needs, E. C., Kelly, A. L., Proteolysis and physicochemical changes in milk on storage as affected by UHT treatment, plasmin activity and KIO_3 addition, Int. Dairy J. 9 (1999) 581-591.

Enright, E., Kelly, A. L., The influence of heat treatment of milk on susceptibility of casein to proteolytic attack by plasmin, Milchwissenschaft 54 (9) (1999) 491-493.

Erbersdobler, H. F., Dehn, B., Nangpal, A., Reuter, H., Determination of furosine in heated milk as a measure of heat intensity during processing, J. Dairy Res. 54 (1987) 147-151.

Erbersdobler, H. F., Dehn-Müller, B., Formation of early Maillard products during UHT treatment of milk, IDF Bulletin 238 (1989) 62-67.

Erbersdobler, H. F., Faist, V., Metabolic transit of Amadori products, Nahrung/Food 45 (3) (2001) 177-181.

Erbersdobler, H. F., Hupe, A., Determination of lysine damage and calculation of lysine bioavailability in several processed foods. Z. Ernährungswiss. 30 (1991) 46-49.

Erbersdobler, H. F., Somoza, V., Forty years of furosine – forty years of using Maillard reaction products as indicators of the nutritional quality of foods, Mol. Nutr. Food Res. 51 (2007) 423-430.

Evangelisti, F., Calcagno, C., Nardi, S., Zunin, P., Deterioration of protein fraction by Maillard reaction in dietetic milks, J. Dairy Res. 66 (2) (1999) 237-243.

Evangelisti, F., Calcagno, C., Zunin, P., Relationship between blocked lysine and carbohydrate composition of infant formulas, J. Food Sci. 59 (2) (1994) 335-337.

Finot, P-A., Chemical modifications of the milk protein during processing and storage. Nutritional, metabolic and physiological consequences. Kieler Milchwirtschaftliche Forschungsberichte 35 (3) (1983) 357-369.

Finot, P-A., Metabolism and physiological effects of Maillard reaction products (MRP). In: Maillard reaction in food processing, human nutrition and physiology (ed. P.A. Finot et al.), Birkenhäuser, Basel 1990, pp 259-272.

Finot, P-A., The absorption and metabolism of modified amino acids in processed foods, J. AOAC Int. 88 (3) (2005a) 894-903.

Finot, P-A., Historical perspective of the Maillard reaction in food science, Ann. N. Y. Acad. Sci. 1043 (2005b) 1-8.

Finot, P. A., Deutsch, R., Bujard, E. The extent of the Maillard reaction during the processing of milk, Prog. Fd. Nutr. Sci. 5 (1981) 345-355.

Finot, P. A., Mauron, J., Le blocage de la lysine par la réaction de Maillard II. Propriétés chimiques des dérivés N-(désoxy-1-D-fructosyl-1) et N-(désoxy-1-D-lactulosyl-1) de la lysine, Helvetica Chimica Acta 55 (1) (1972) 1153-1164.

Flynn, R. G., Bakal, A. I., Snyder, M. A., Method of preparing lactose-hydrolysed milk with suppressed sweetness, US5334399 (1994).

Forsman, E-S., Heikonen, M., Kiviniemi, L., Kreula, M., Linko, P., Kinetic investigations of the hydrolysis of milk lactose with soluble *Kluyveromyces lactis* β-galactosidase, Milchwissenschaft 34 (10) (1979) 618-621.

Fox, P.F., Kelly, A. L., Indigenous enzymes in milk: Overview and historical aspects – Part 1, Int. Dairy J. 16 (2006) 500-516.

Frank, J.F., Christen, G.L., Bullerman, L.B., Tests for groups of microorganisms. In: Standard Methods for the Examination of Dairy Products (ed. R.T. Marshall) 16th ed., s. 271-286. APHA, Washington, DC 1992. Valio microbiological methods 7 - thermophilic bacteria.

Friedman, M., Improvement in the safety of foods by SH-containing amino acids and peptides. A review, J. Agric. Food Chem. 42 (1994) 3-20.

Friedman, M., Food browning and its prevention: An overview, J. Agric. Food Chem. 44 (3) (1996) 631-653.

Friedman, M., Finot, P. A., Nutritional improvement of bread with lysine and γ-glutamyllysine, J. Agric. Food Chem. 38 (1990) 2011-2020.

Friedman, M., Molnar-Perl, I., Inhibition of browning by sulfur amino acids. 1. Heated amino-acid-glucose systems, J. Agric. Food Chem. 38 (1990) 1642-1647.

Furniss, D. E., Vuichoud, J., Finot, P. A., Hurrell, R. F., The effect of Maillard reaction products on zinc metabolism in the rat, Br. J. Nutr. 62 (3) (1989) 739-749.

Geciova, J., Bury, D., Jelen, P., Methods for disruption of microbial cells for potential use in the dairy industry – a review, Int. Dairy J. 12 (2002) 541-553.

Gerhardinger, C., Marion, M. S., Rovner, A., Glomb, M., Monnier, V. M., Novel degradation pathway of glycated amino acids into free fructosamine by *Pseudomonas* sp. soil strain extract, J. Biol. Chem. 270 (1) (1995) 218-224.

Goodno, C. C., Swaisgood, H. E., Catignani, G. L., A fluorimetric assay for available lysine in proteins, Anal. Biochem. 115 (1981) 203-211.

Greenberg, N. A., Mahoney, R. R., Immobilisation of lactase (beta-galactosidase) for use in dairy processing: a review, Process Biochem. 16 (2) (1981) 2-8.

Grufferty, M. B., Fox, P. F., Potassium iodate induced proteolysis in ultra high treated milk during storage: The role of β-lactoglobulin and plasmin, J. Dairy Res. 53 (1986) 601-613.

Guamis, B., Huerta, T., Garay, E., Heat-inactivation of bacterial proteases in milk before UHT-treatment, Milchwissenschaft 42 (10) (1987) 651-653.

Harju, M., Microbiological control of an immobilized enzyme reactor, Nordeuropæisk Mejeri-tidskrift 43 (1977) 155-159.

Harju, M., Lactose hydrolysis, IDF Bulletin 212 (1987a) 50-55.

Harju, M., A method for the spesific separation of lactose from skim milk, Finnish J. Dairy Sci. 45 (1) (1987b) 82-93.

Harju, M., A process for the specific separation of lactose from milk, EP0226035 (1990).

Harju, M., Chromatographic and enzymatic removal of lactose from milk, IDF Bulletin 389 (2004) 4-8.

Harju, M., Heikkilä, H., Process for recovering lactose from whey. US Patent 4.955.363 (1990).

Hayward, L. D. and Angyal, S. J. A symmetry rule for the circulair dicroism of reducing sugars, and the proportion of carbonyl forms in aqueous solutions thereof. Carbohydr. Res. 53 (1977) 13-20.

Henle, T., Walter, H., Klostermeyer, H., Evaluation of the extent of the early Maillard-reaction in milk products by direct measurement of the Amadori-product lactuloselysine, Z. Lebensm. Unters. Forsch. 193 (1991) 119-122.

Hinrichs, J., Kessler, H. G., Thermal processing of milk – processes and equipment. In: Heat-induced changes in milk, 2nd ed., IDF, Brussels 1995, 9-21.

Holsinger, V. H., Kligerman, A. E. Applications of lactase in dairy foods and other foods containing lactose, Food Technol. 45 (1) (1991) 92, 94-95.

Horak, F. P., Kessler, H. G., The influence of UHT heating and sterilization on lysine in milk, Milchwissenschaft 36 (9) (1981) 543-547.

Hurrell, R. F., Influence of the Maillard reaction on the nutritional value of foods. In: Maillard reaction in food processing, human nutrition and physiology (ed. P.A. Finot et al.), Birkenhäuser, Basel 1990, pp 245-258.

Hurrell, R. F., Carpenter, K. J., The estimation of available lysine in foodstuffs after Maillard reactions, Prog. Fd Nutr. Sci. 5 (1981) 159-176.

Hurrell, R. F., Finot, P. A., Food processing and storage as a determinant of protein and amino acid availability, Experientia Suppl. 44 (1983) 135-156.

Hurrell, R. F., Finot, P. A., Ford, J. E., Storage of milk powders under adverse conditions. 1. Losses of lysine and of other essential amino acids as determined by chemical and microbiological methods, Br. J. Nutr. 49 (1983) 343-354.

Hurrell, R. F., Lerman, P., Carpenter, K. J., Reactive lysine in foodstuffs as measured by a rapid dye-binding procedure, J. Food Sci. 44 (1979) 1221-1227, 1231.

Hyrkäs, K., Viskari, R., Linko, Y-Y., Linko, M., Hydrolysis of lactose in acid whey by immobilized β-galactosidase, Milchwissenschaft 31 (3) (1976) 129-134.

IDF 1D:1996. Milk – Determination of fat content – Röse-Gottlieb gravimetric method (Reference method).

IDF 20-1/ISO 8968-1 (2001). Milk -Determination of nitrogen content, Part 1: Kjeldahl method.

IDF 21B:1987 modified. Valio R&D/Chemistry 10. Dry-matter, fast. Valio R&D/Chemistry method 10.

IDF 73B:1998. Enumeration of coliforms, Part 1: Colony count technique at 30°C without resuscitation (VRB-agar method).

IDF 99C:1997 – Sensory evaluation of dairy products by scoring – Reference method.

IDF 154:1992, NMKL 173:2005 modified Valio R&D/Chemistry method 50 – Ash.

IDF 193:2004(E)/ISO 18329:2004(E). Milk and milk products – Determination of furosine content – Ion-pair reverse-phase high-performance liquid chromatography method, 11 p.

ISO 4833:2003 – Standard plate count.

ISO 7932:2004 modified. Valio microbiological methods 14a – *Bacillus cereus*.

ISO 17410:2001 Valio microbiological methods 6 – psychrotrophic micro-organisms.

Izzo, H. V., Ho, C-T., Peptide-specific Maillard reaction products: a new pathway for flavor chemistry, Trends in Food Sci. Technol. 3 (1992) 253-257.

Jelen, P., Tossavainen, O., Low lactose and lactose-free milk and dairy products – prospects, technologies and applications, Aust. J. Dairy Technol. 58 (2) (2003) 161-165.

Jeon, I. J., Mantha, V. R., High performance liquid chromatography analysis of oligosaccharides formed during β-galactosidase action on lactose, J. Dairy Sci. 68 (1985) 581-588.

Kaminogawa, S., Totsuka, M., Allergenicity of milk proteins. In: Advanced Dairy Chemistry Volume 1: Proteins (eds. P. F. Fox, P.L.H. McSweeney), 3rd ed., Kluwer Academic /Plenum Publishers, New York 2003, pp. 651-674.

Kelly, A. L., McSweeney, P. L. H., Indigenous proteinases in milk. In: Advanced dairy chemistry, Vol 1: Proteins (eds. P. F. Fox, P. L. H. McSweeney), 3rd ed., Kluwer Academic /Plenum Publishers, New York 2003, pp. 495-521.

Kelly, A. L., O'Flaherty, F., Fox, P.F., Indigenous proteolytic enzymes in milk: A brief overview of the present state of the knowledge, Int. Dairy J. 16 (2006) 563-572.

Kessler, H. G., Milchbearbeitung. In: Handbuch Milch (ed. E. Hetzner), Behr's Verlag 1992.

Kessler, H. G., Heat treatment, processes and effects - microorganisms and conditions of inactivation. In: Food and Bio Process Engineering – Dairy Technology, Verlag A. Kessler, München 2002, pp. 130-147.

Kocak, H. R., Zadow, J. G., Changes in the characteristics of lactose hydrolysed UHT milk during storage, Proc. XXI International Dairy Congress Vol. 1, book 2, brief communications, Moscow 12-16.7.1982, 209.

Kocak, H. R., Zadow, J. G., The effect of low-temperature-inactivation on age gelation of UHT whole milk, Aust. J. Dairy Technol. 40 (1985a) 53-58.

Kocak, H. R., Zadow, J. G., Age gelation of UHT whole milk as influenced by storage temperature, Aust. J. Dairy Technol. 40 (1) (1985b) 14-21.

Kocak, H. R., Zadow, J. G., The effect of lactose hydrolysis and subsequent low-temperature-inactivation on age gelation of UHT whole milk, Aust. J. Dairy Technol. 44 (1989) 37-40.

Kroh, L., Zeise, S., Stösser, R., Westphal, G., Untersuchungen zur Maillard-Reaktion. 18. Mitteilung. Radikalische Inhibierung der Bräunung durch Schwefelverbindungen, Z. Lebensm. Untersuch. Forsch. 188 (1989) 115-117.

Kuokkanen, M., Enattah, N. S., Oksanen, A., Savilahti, E., Orpana, A., Järvelä, I., Transcriptional regulation of the lactase-phlorizin hydrolase gene by polymorphisms associated with adult-type hypolactasia, Gut 52 (2003) 647-652.

Labuza, T. P., Saltmarch, M. The nonenzymatic browning reaction as affected by water in foods. In: Water activity: Influences on food quality (eds. B. Rockland, G. F. Stewart), Academic Press, New York 1981, pp. 605-650.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (1970) 680-685.

Lange, M., Lactose-free milk and process for making same. US 2005/0170044A1 (2005).

Leclère, J., Birlouez-Aragon, I., The fluorescence of advanced Maillard products is a good indicator of lysine damage during the Maillard reaction, J. Agric. Food Chem. 49 (2001) 4682-4687.

Leitner, G., Krifucks, O., Merin, U., Lavi, Y., Silanikove, N., Interactions between bacteria type, proteolysis of casein and physico-chemical properties of bovine milk, Int. Dairy J. 16 (2006) 648-654.

Lieske, B., Konrad, G., Eine kolorimetrische Routinemethode zur quantitativen Mikroanalyse von freier α-Aminostickstoff in Proteinhydrolysaten, Nahrung 21 (10) (1977) 925-930.

López-Fandiño, R., Olano, A., San José, C., Ramos, M., Application of reverse-phase HPLC to the study of proteolysis in UHT milk, J. Dairy Res. 60 (1993) 111-116.

Mahoney, R. R., Lactose: enzymatic modification. In: Advanced Dairy Chemistry Volume 3: Lactose, water, salts and vitamins (Ed. P. F. Fox), 2nd ed., Chapman & Hall, London 1997 pp. 77-125.

Mahoney, R. R., Galactosyl-oligosaccharide formation during lactose hydrolysis: a review, Food Chemistry 63 (2) (1998) 147-154.

Manji, B., Kakuda, Y., Arnott, D. R., Effect of storage temperature on age gelation of ultra-high temperature milk processed by direct and indirect heating systems, J. Dairy Sci. 69 (1986) 2994-3001.

Manzi, P., Marconi, S., Pizzoferrato, L., New functional milk-based products in the Italian market, Food Chemistry 104 (2007) 808-813.

Marconi, E., Messia, M. C., Panfili, G., Characterization of lactose-hydrolysed milks by using various process and product indicators. In Proceedings of Congrilait 2002, 26th IDF World Dairy Congress, Paris, France, Sept. 24-27.2002; IDF, Brussels, Belgium 2002.

Marteau, P., Morales, E., Vesa, T., Korpela, R., Lactose intolerance: the medical point of view, Sciences des Aliments 22 (2002) 431-436.

Marteau, P., Vesa, T. H., Korpela, R., Lactose maldigestion and intolerance. Proceedings of 25th International Dairy Congress, Aarhus, Denmark, IDF 1998. Part: Milk and health, pp. 78-87.

Matsubara, H., Hagihara, B., Nakai, M., Komaki, T., Yonetani, T., Okunuki, K., Crystalline bacterial proteinase II. General properties of crystalline proteinase of *Bacillus subtilis* N', J. Biochem. 45 (4) (1958) 251-258.

Matthews, B. W., The structure of *E. coli* β-galactosidase, C. R. Biologies 328 (2005) 549-556.

McKellar, R. C., Development of off-flavours in ultra-high temperature and pasteurized milk as a function of proteolysis, J. Dairy Sci. 64 (1981) 2138-2145.

Mendoza, M. R., Olano, A., Villamiel, M., Chemical indicators of heat treatment in fortified and special milks, J. Agric. Food Chem. 53 (2005) 2995-2999.

Messia, M. C., Candigliota, T., Marconi, E., Assessment of quality and technological characterization of lactose-hydrolysed milk, Food Chemistry 104 (2007) 910-917.

Metwalli, A. A. M., de Jongh, H. H. J., van Boekel, M. A. J. S., Heat inactivation of bovine plasmin, Int. Dairy J. 8 (1998) 47-56.

Miller, J. J., Brand, J. C., Enzymic lactose hydrolysis, Food Technol. Aust. 32 (3) (1980) 144-147.

Miranda, G., Gripon, J. C., Origine, nature et incidences technologiques de la protéolyse dans le lait, Lait 66 (1986) 1-18.

Mitchell, I. R., Hourigan, J. A., Kinetics of lactose hydrolysis and uses for lactose hydrolysed products, IDF Bulletin 289 (1993) 31-32.

Mittal, S.B., Hourigan, J. A., Zadow, J. G., Effect of UHT treatment, storage time and temperature on the available lysine of UHT recombined and UHT recombined lactose hydrolysed milk, Aust. J. Dairy Technol. 44 (1989) 88-90.

Mittal, S. B., Hourigan, J. A., Zadow, J. G., Nguyen, M. H., Behaviour of UHT recombined and UHT recombined lactose hydrolysed milk during storage at different temperatures, Aust. J. Dairy Technol. 43 (2) (1988) 64-73.

Mittal, S. B., Newell, G., Hourigan, J. A., Zadow, J. G., The effect of protease contamination in lactase on the flavour of lactose-hydrolysed milks, Aust. J. Dairy Technol. 46 (1991) 46-47.

Modler, H. W., Gelda, A., Yaguchi, M., Gelda, S., Production of fluid milk with a high degree of lactose hydrolysis, IDF Bulletin 289 (1993) 57-61.

Molnar-Perl, I., Friedman, M., Inhibition of browning by sulfur amino acids. 2. Fruit juices and protein containing foods, J. Agric. Food Chem. 38 (1990) 1648-1651.

Morales, F. J., van Boekel, M. A. J. S., A study on advanced Maillard reaction in heated casein/sugar solutions: colour formation, Int. Dairy J. 8 (1998) 907-915.

Moreaux, V., Birlouez-Aragon, I., Degradation of tryptophan in heated β-lactoglobulin-lactose mixtures is associated with intense Maillard reaction. J. Agric. Food Chem. 45 (1997) 1905-1910.

Morisi, F., Pastore, M., Viglia, A., Reduction of lactose content of milk by entrapped β-galactosidase I. Characteristics of β-galactosidase from yeast and *Escherichia coli*, J. Dairy Sci. 56 (1973) 1123-1127.

Mozaffar, Z., Nakanishi, K., Matsuno, R., Kamikubo, T., Purification and properties of β-galactosidases from *Bacillus circulans*, Agric. Biol. Chem. 48 (1984) 3053-3061.

Mustapha, A., Herzler, S. R., Savaiano, D. A., Lactose: Nutritional significance. In: Advanced Dairy Chemistry Volume 3: Lactose, water, salts and vitamins (Ed. P. F. Fox), 2nd ed., Chapman & Hall, London 1997 pp. 127-154.

Möller, A. B., Andrews, A. T., Cheeseman, G. C., Chemical changes in ultra-heat-treated milk during storage. I. Hydrolysis of casein by incubation with pronase and a peptidase mixture, J. Dairy Res. 44 (1977a) 259-266.

Möller, A. B., Andrews, A. T., Cheeseman, G. C., Chemical changes in ultra-heat-treated milk during storage, II. Lactuloselysine and fructoselysine formation by the Maillard reaction, J. Dairy Res. 44 (1977b) 267-275.

Nakao, M., Harada, M., Kodama, Y. Nakayama, T., Shibano, Y., Amachi, T., Purification and characterization of a thermostable β-galactosidase with high transgalactosylation activity from *Saccharopolyspora rectivirgula*. Appl. Microbiol. Biotechnol. 40 (1994) 657-663.

Nangpal, A., Reuter, H., Dehn-Müller, B., Erbersdobler, H., Formation of furosine during UHT treatment of milk – comparison between direct and indirect heating, Kieler Milchwirtschaftliche Forschungsberichte 42 (1) (1990) 43-51.

Naranjo, G. B., Malec, L. S., Vigo, M. S., Reducing sugars effect on available lysine loss of casein by moderate heat treatment, Food Chem. 62 (3) (1998) 309-313.

NDC, Nutritional implications of lactose and lactase activity, Dairy Council Dig. 56 (5) (1985) 25.

Neuhaus, W., Novalin, S., Klimacek, M., Splechtna, B., Petzelbauer, I., Szivak, A., Kulbe, K. D., Optimization of an innovative hollow-fiber process to produce lactose- reduced skim milk, Appl. Biochem. Biotechnol. 134 (1) (2006) 1-14.

Newstead, D. F., Paterson, G., Anema, S. G., Coker, C. J., Wewala, A. R., Plasmin activity in direct-steam-injection UHT-processed reconstituted milk: Effects of preheat treatment, Int. Dairy J. 16 (2006) 573-579.

Nieuwenhuijse, J. A., Changes in heat-treated milk products during storage. In: Heat- induced changes in milk (ed. P. F. Fox), IDF, Brussels 1995, pp. 231-255.

Nieuwenhuijse, J. A., van Boekel, M. A. J. S., Protein stability in sterilized milk and milk products. In: Advanced Dairy Chemistry Volume 1: Proteins (eds. P. F. Fox, P. L. H. McSweeney), 3rd ed., Kluwer Academic/Plenum Publishers, New York 2003, pp. 947-974.

Novalin, S., Neuhaus, W., Kulbe, K. D., A new innovative process to produce lactose-reduced skim milk, J. Biotechnology 119 (2005) 212-218.

Nursten, H. E., Recent development in studies of the Maillard reaction. Food Chem. 6 (1981) 263-277.

O'Brien, J., Heat-induced changes in lactose isomerization, degradation, Maillard browning. In: Heat-induced chances in milk (Ed. P.F. Fox), 2nd ed., IDF, Brussels 1995, 134-170.

O'Brien, J., Reaction chemistry of lactose: non-enzymatic degradation pathways and their significance in dairy products. In Advanced Dairy Chemistry, Volume 3: Lactose, water, salts and vitamins (Ed. P. F. Fox), 2nd ed., Chapman & Hall, London 1997, 155-231.

O'Brien, J. M., Morrissey, P. A., The Maillard reaction in milk products, IDF Bulletin 238 (1989a) 53-61.

O'Brien, J. M., Morrissey, P. A., Nutritional and toxicological aspects of the Maillard browning reaction in foods, Crit. Rev. Food Sci. Nutr. 28 (3) (1989b) 211-248.

Pagliarini, E., Vernile, M., Peri, C., Kinetic study on color changes in milk due to heat, J. Food Sci. 55 (6) (1990) 1766-1767.

Palomaa, H., Fullständig enzymatic hydrolys av laktos, pro gradu, Helsingfors Universitet, Institutionen för livsmedelsteknologi, 2001, 74 p.

Panesar, P. S., Panesar, R., Singh, R. S., Kennedy, J. F., Kumar, H., Microbial production, immobilization and applications of β-D-galactosidase, J Chem Technol Biotechnol 81 (2006) 530-543.

Pastore, M., Morisi, F., Zaccardelli, D., Reduction of lactose content of milk using entrapped β-galactosidase III. Pilot-plant experiments. In: Insolubilized enzymes (eds. M. Salmona, C. Saronio, S. Garattini), Raven Press, New York 1974, pp. 211-216.

Pellegrino, L., Resmini, P., Luf, W., Assessment (indices) of heat treatment of milk. In Heat-induced chances in milk (Ed. P.F. Fox), 2.ed., IDF, Brussels 1995, 409-453.

Perkins, M. L., Elliott, A. J., D'Arcy, B. R., Deeth, H. C., Stale flavour volatiles in Australian commercial UHT milk during storage, Aust. J. Dairy Technol. 60 (3) (2005) 231-237.

Peterson, G. L., Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall, Anal. Biochem. 100 (1979) 201-220.

Petzelbauer, I., Kuhn, B., Splechtna, B., Kulbe, K. D., Nidetzky, B., Development of an ultrahigh-temperature process for the enzymatic hydrolysis of lactose. IV. Immobilization of two thermostable beta-glycosidases and optimization of a packed-bed reactor for lactose conversion, Biotechn. Bioeng. 77 (6) (2002b) 619-631.

Petzelbauer, I., Nidetzky, B., Haltrich, D., Kulbe, K. D., Development of an ultra-high-temperature process for the enzymatic hydrolysis of lactose. I. The properties of two thermostable β-glycosidases, Biotechn. Bioeng. 64 (3) (1999) 322-332.

Petzelbauer, I., Splechtna, B., Nidetzky, B., Development of an ultrahigh-temperature process for the enzymatic hydrolysis of lactose. III. Utilization of two thermostable beta-glycosidases in a continuous ultrafiltration membrane reactor and galacto-oligosaccharide formation under steady state conditions, Biotechn. Bioeng. 77 (4) (2002a) 394-404.

Petzelbauer, I., Zeleny, R., Reiter, A., Kulbe, K. D., Nidetzky, B., Development of an ultra-high-temperature process for the enzymatic hydrolysis of lactose. II. Oligosaccharide formation by two thermostable beta-glycosidases, Biotechn. Bioeng. 69 (2) (2000) 140-149.

Prado, B. M., Ismail, B., Ramos, O., Hayes, K. D., Thermal stability of plasminogen activators and plasminogen activation in heated milk, Int. Dairy J. 17 (2007) 1028-1033.

Prado, B. M., Sombers, S. E., Ismail, B., Hayes, K. D., Effect of heat treatment on the activity of inhibitors of plasmin and plasminogen activators in milk, Int. Dairy J. 16 (2006) 593-599.

Rehner, G., Walter, T., Wirkung von Maillard-Produkten und Lysinolalanin auf die Bioverfügbarkeit von Eisen, Kupfer and Zink, Z. Ernährungswiss. 30 (1991) 50-55.

Renner, E., Fetter, C. H., Renz-Schauen, A., Einfluss der Lactosehydrolyse auf Qualitätskriterien von UHT-Milch, Meijeritieteellinen Aikakausikirja XLIV (1) (1986) 12-22.

Renz-Schauen, A., Nutritional value of protein in lactose-hydrolysed UHT milk, Kieler Milchwirtschaftliche Forschungsberichte 35 (3) (1983) 325-327.

Rérat, A., Calmes, R., Vaissade, P, Finot, P-A., Nutritional and metabolic consequences of the early Maillard reaction of the heat treated milk in the pig. Significance for man, Eur. J. Nutr. 41 (2002) 1-11.

Resmini, P., Pellegrino, L., Battelli, G., Accurate quantification of furosine in milk and dairy products by a direct HPLC method, Ital. J. Food Sci (3) (1990) 173-183.

Resmini, P., Pellegrino, L., Masotti, F., Evaluation of the extent of the Maillard reaction for the quality control of low-heat-treated dairy products, IDF Special issue, 9303 (1993) 153-164.

Richmond, M. L., Gray, J. I., Stine, C. M., Beta-galactosidase: Review of recent research related to technological application, nutritional concerns, and immobilization, J. Dairy Sci. 64 (1981) 1759-1771.

Rollema, H.S., Poll, J. K., The alkaline milk proteinase system: kinetics and mechanism of heat-inactivation, Milchwissenschaft 41 (9) (1986) 536-540.

Rufián-Henares, J. A., García-Villanova, B., Guerra-Hernández, E., Furosine content, loss of o-phtaldialdehyde reactivity, fluorescence and colour in stored enteral formulas, Int. J. Dairy Technol. 55 (3) (2002) 121-126.

Rufián-Henares, J. A., García-Villanova, B., Guerra-Hernández, E., Generation of furosine and color in infant/enteral formula-resembling systems, J. Agric. Food Chem. 52 (2004) 5354-5358.

Rysstad, G., Kolstad, J., Extended shelf life milk - advances in technology, Int. J. Dairy Technol. 59 (2) (2006) 85-96.

Sahi, T., Hypolactasia and lactase persistence, Historical review and the terminology, Scand. J. Gastroenterol. 202 (29 Suppl) (1994a) 1-6.

Sahi, T., Genetics and epidemiology of adult-type hypolactasia, Scand. J. Gastroenterol. 202 (29 Suppl) (1994b) 7-20.

Saint Denis, T., Humbert, G., Gaillard, J-L., Heat inactivation of native plasmin, plasminogen and plasminogen activators in bovine milk: a revisited study, Lait 81 (2001) 715-729.

Samel, R., Weaver, R. W. V., Gammack, D. B., Changes on storage in milk processed by ultra-high-temperature sterilization, J. Dairy Res. 38 (1971) 232-332.

Savaiano, D. A., Lactose maldigestion vs. intolerance, Sciences des Aliments 22 (2002) 425-430.

Schaafsma, G., Effects of heat treatment on the nutritional value of milk, IDF Bulletin 238 (1989) 68-70.

Schamberger, G. P., Labuza, T. P., Effect of green tea flavonoids on Maillard browning in UHT milk, LWT 40 (2007) 1410-1417.

Sherr, B., Lee, C. M. Jelesciewicz, C., Absorption and metabolism of lysine Maillard products in relation to utilization of L-lysine, J. Agr. Food Chem. 37 (1989) 119-122.

Shukla, T. P., Beta-galactosidase technology: a solution to the lactose problem, CRC Crit. Rev. Food Technol. 5 (1975) 325-356.

Silfverberg, P., Tossavainen, O., Jonson, V., Menetelmä vähälaktoosisten ja laktoosittomien hapanmaitotuotteiden valmistamiseksi, FI118115B (2007).

Simoons, E. J., The geographic hypothesis and lactose malabsorption. A weighing of the evidence, Digestive Diseases 23 (11) (1978) 963-980.

Sloan, A. E., The new market: Foods for the not-so-healthy, Food Technology 53 (2) (1999) 54-60.

Smart, J. B., Transferase reactions of β-galactosidases – new product opportunities, IDF Bulletin 289 (1993) 16-22.

Snoeren, T. H. M., Both, P., Proteolysis during the storage of UHT-sterilized whole milk. 2. Experiments with milk heated by the indirect system for 4 s at 142°C, Neth. Milk Dairy J. 35 (1981) 113-119.

Snoeren, T. H. M., van der Spek, C. A., Dekker, R., Both, P., Proteolysis during the storage of UHT-sterilized whole milk. 1. Experiments with milk heated by the direct system for 4 seconds at 142°C, Neth. Milk Dairy J. 33 (1979) 31-39.

Somkuti, G. A., Dominiecki, M. E., Steinberg, D. H., Permeabilization of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* with ethanol, Curr. Microbiol. 36 (1998) 202-206.

Stepaniak, L., Sørhaug, T., Thermal denaturation of bacterial enzymes in milk. In: Heat-induced chances in milk (ed. P.F. Fox), 2.ed., IDF, Brussels 1995, 349-363.

Swaisgood, H. E., Enzymes indigenous to bovine milk. In: Handbook of milk composition (ed. R. G. Jensen), Academic Press, New York 1995, pp. 472-476.

Swallow, D. M., Harvey, C. B., Genetics of adult-type hypolactasia. In: Common Food Intolerances 2: Milk in Human Nutrition and Adult-Type Hypolactasia (eds. S. Auricchio and G. Semenza), Karger, Basel 1993, pp. 85-92.

Sørhaug, T., Stepaniak, L., Microbial enzymes in the spoilage of milk and dairy products. In: Food Enzymology, Vol. 1. (ed. P. F. Fox), Elsevier Appl. Sci., London 1991, 169-218.

Tamura, K., Akitaya, M., Honda, Y., Ahiko, K., Preparation of low sweetness lactose-hydrolysis milk and its effects when fed to constipated or lactose intolerant individuals, Reports of Research Laboratory Technical Research Institute Snow Brand Milk Products Co. 95 (1991) 61-69. Abstract.

TetraPak International, UK Patent 1.477.087 (1977).

Thompkinson, D. K., Immobilization of β-galactosidase on macroporous anion exchange resin, IDF Bulletin 289 (1993) 23-26.

Thompkinson, D. K., Mathur, B. N., Rajore, R. B., Process alternatives for lactose hydrolysis, Indian dairyman 43 (9) (1991) 394-402.

Topçu, A., Numanoğlu, E., Saldamlı, I., Proteolysis and storage stability of UHT milk produced in Turkey, Int. Dairy J. 16 (2006) 633-638.

Tossavainen, O., Sahlstein, J., Process for producing a lactose-free milk product, EP1503630B1 (2003).

Valero, E., Villamiel, M., Miralles, B., Sanz, J., Martínez-Castro, I., Changes in flavour and volatile components during storage of whole and skimmed UHT milk, Food Chem. 72 (2001) 51-58.

van Boekel, M.A.J.S., Kinetic modeling of sugar reactions in heated milk-like systems, Neth. Milk Dairy J. 50 (1996) 245-266.

van Boekel, M.A.J.S., Kinetic aspects of Maillard reaction: a critical review, Nahrung/Food 45 (3) (2001) 150-159.

Van Dam, B., Revallier-Warffemius, J. G., Van Dam-Schermerhorn, L. C., Preparation of lactase from *Saccharomyces fragilis*, Neth. Milk Dairy J. 4 (1950) 96-114.

Van Renterghem, R., De Block, J., Furosine in consumption milk and milk powders, Int. Dairy J. 6 (1996) 371-382.

Vasala, A., Huumonen, J., Alatossava, T., Menetelmä maitotuotteen makeuden peittämiseksi, FI100375B (1996).

Vasiljevic, T., Jelen, P., Production of β-galactosidase for lactose hydrolysis in milk and dairy products using thermophilic lactic acid bacteria, Innov. Food Sci. & Emerg. Tech. 2 (2001) 75-85.

Vasiljevic, T., Wismer, W., Jelen, P., Sensory effects of lactose hydrolysis in milk by crude cellular extracts from *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842, Milchwissenschaft 58 (3/4) (2003) 167-170.

Vigo, M. S., Malec, L. S., Gomez, R. G., Llosa, R. A., Spectrophotometric assay using o-phthaldialdehyde for determination of reactive lysine in dairy products, Food Chem. 44 (1992) 363-365.

Vis, E. H., Plinck, A. F., Alink, G. M., van Boekel, M. A. J. S., Antimutagenicity of heat denatured ovalbumin, before and after digestion, as compared to caseinate, BSA, and soy protein, J. Agric. Food Chem. 46 (1998) 3713-3718.

Walstra, P., Wouters, J. T. M., Geurts, T. J., Dairy Science and Technology, 2. ed., Taylor & Francis Group, Boca Raton 2006, p. 440.

Wang, J., Lactose-removed milk product and process for the preparation thereof, US 2005/0196508 A1 (2005).

Watanabe, N., Ohtsuka, M., Takahashi, S.-I., Sakano, Y., Fujimoto, D., Enzymatic deglycation of fructosyl-lysine, Agric. Biol. Chem. 54 (4) (1990) 1063-1064**.**

Woychik, J. H., Wondolowski, M. V., Dahl, K. J., Preparation and application of immobilized β-galactosidase of *Saccharomyces lactis.* In: Immobilized enzymes in food and microbial processes (eds. A. C. Olson, C. L. Cooney), Plenum Press, New York 1974, pp. 41-49.

Zadow, J. G., Lactose: Properties and uses, J. Dairy Sci. 67 (1984) 2654-2679.

Zadow, J. G., Lactose hydrolysed dairy products, Food Technol. Aust. 38 (11) (1986) 460-462, 471.

Zadow, J. G., Economic considerations related to the production of lactose and lactose by-products, IDF Bulletin 289 (1993) 10-15.

Zadow, J. G., Birtwistle, R., The effect of dissolved O_2 on the changes occurring in the flavour of ultra-high-temperature milk during storage, J. Dairy Res. 40 (1973) 169-177.

Zárate, S., López-Leiva, M. H., Oligosaccharide formation during enzymatic lactose hydrolysis: A literature review, J. Food Prot. 53 (3) (1990) 232-268.

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